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CHARACTERISATION AND SUSCEPTIBILITY TO LETHAL PHOTOSENSITISATION OF *IN VITRO* – GROWN SUB – GINGIVAL BIOFILMS

Thesis submitted by

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DECLARATION

I hereby certify that the work embodied in this thesis is the result of my own investigation, except where otherwise stated. Transmission and Scanning Electron Micrograph processing was carried out by Mrs Nicola Mordan of the Electron Microscopy Unit of the Eastman Dental Institute. Confocal Microscopy was carried out in conjunction with Dr Christopher Hope of the Eastman Dental Institute and Daniel Ciantar of the Anatomy Department, University College London.

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Abstract

Oral biofilms are the aetiological agents of periodontal diseases. Biofilms are inherently resistant to traditional antimicrobials, hence novel treatment modalities, like photodynamic therapy (PDT), might be an efficacious alternative to traditional antimicrobials. Preliminary PDT studies involved the screening of a number of photosensitisers, Toluidine blue O (TBO) was chosen for further investigation. TBO in conjunction with a 35mW Helium Neon (HeNe) laser was then tested against a range of planktonic oral bacteria as well as both monospecies biofilms grown on membrane filters and multi-species biofilms grown on membrane filters from a salivary inoculum. TBO achieved appreciable kills against many of the planktonic organisms, whilst a 2 $\log_{10}$ reduction was evident in both the monospecies biofilms and the saliva-derived biofilms following irradiation with 31.5 J of laser light in the presence of 81.7 μM TBO. In order to evaluate the likely efficacy of PDT *in vivo* it was necessary to develop a laboratory model that was capable of producing biofilms that would resemble (in terms of composition and structure) the subgingival plaques found in periodontitis patients *in vivo*. Consequently, biofilms were grown from subgingival plaques in a Constant Depth Film Fermentor (CDFF) under conditions mimicking those present within a periodontal pocket. The resultant biofilms were quantified using viable counts on selective agars and the microbiota was characterised using 16S rRNA gene sequencing. The community structure was analysed by denaturing gradient gel electrophoresis and community level physiological profiling. The structure of the biofilms was studied in real time using confocal scanning laser microscopy. The susceptibility to PDT (using toluidine blue and 632 nm light)

of these subgingivally-derived biofilms was then determined. Reductions in viability of up to 1 $\log_{10}$ were observed and when compared to chlorhexidine digluconate (an antimicrobial commonly used for the treatment of oral infections and often considered to be the "gold standard"), there was little difference in efficacy. In this study, a laboratory model able to produce biofilms with a composition and structure resembling those of subgingival dental plaques was developed. The biofilms produced were found to be susceptible to lethal photosensitisation using toluidine blue and light (632 nm).

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CHAPTER ONE

INTRODUCTION

1.1 Biofilms

Biofilms are one of the most ubiquitous microbiological phenomena in the natural world. The first tentative analysis of what was later to be termed a biofilm was carried out by Antonie van Leuwenhoek over three hundred years ago when he described the 'animalcules' which were observed from the scrapings taken from a human tooth surface (Costerton, 1999a). One of the earliest techniques utilised to study sessile micro-organisms involved the exposure of glass slides to a variety of differing environments whereupon any microbes present would adhere to the glass surface allowing analysis by microscopy (Jones et al., 1969, Wimpenny et al., 2000). Among the scientists to first study biofilms in any great detail was Claude Zobell who analysed populations derived from an aquatic environment using a light microscope, one of his conclusions being that bacteria are attracted to surfaces whereupon a nascent sessile community may establish (Costerton, 1999a). One of the unique characteristics of a biofilm is the inherent diversity evident upon examination. Biofilms may be composed of many differing bacterial taxa, many of which may require growth conditions which are solely generated within the confines of the biofilm matrix.

1.1.1 Biofilm structure

Biofilms at the simplest and most functional level may be described as accretions of microbes surrounded by a complex coating of polysaccharides, proteins, DNA, RNA, charged ions and water (Sutherland, 2001, Wilson, 2001). However the main component of any biofilm is water which may account for up to 97% of the biofilm matrix (Zhang et al., 1998). Biofilms are

diverse and the above components may vary greatly as a proportion of any given biofilm. Although biofilms had been studied intermittently for the past hundred years, resolving the three-dimensional structure of biofilms proved elusive. Electron microscopy revealed the morphotype of bacteria present and the way in which they were juxtaposed, however it was widely accepted that the sample preparation method used resulted in the visualisation of artefacts and not what may be regarded as the structure present *in vivo*. The elucidation of biofilm structure has been greatly facilitated by the introduction of confocal laser scanning microscopy (CLSM), allowing the examination of the microscopic structures in real time with minimal sample preparation (Cummins et al., 1992). This new technique revealed that the common consensus as to biofilm structure, that of cells in a closely compacted mesh of extracellular polysaccharide (EPS), essentially a rather dense homogeneous configuration lacking any discernible structure, was not a true representation. Biofilms are composed of discrete microcolonies of bacterial cells enclosed within bacterially derived EPS (as shown in figure 1.1), each species within the micocolony occupying a niche unique to its particular biochemical and physiological needs (Costerton, 1995). Moreover, this microcolony arrangement is interspersed with channels facilitating mass transport, waste disposal and gaseous distribution (de Beer et al., 1993, Massol-Deya et al., 1995).

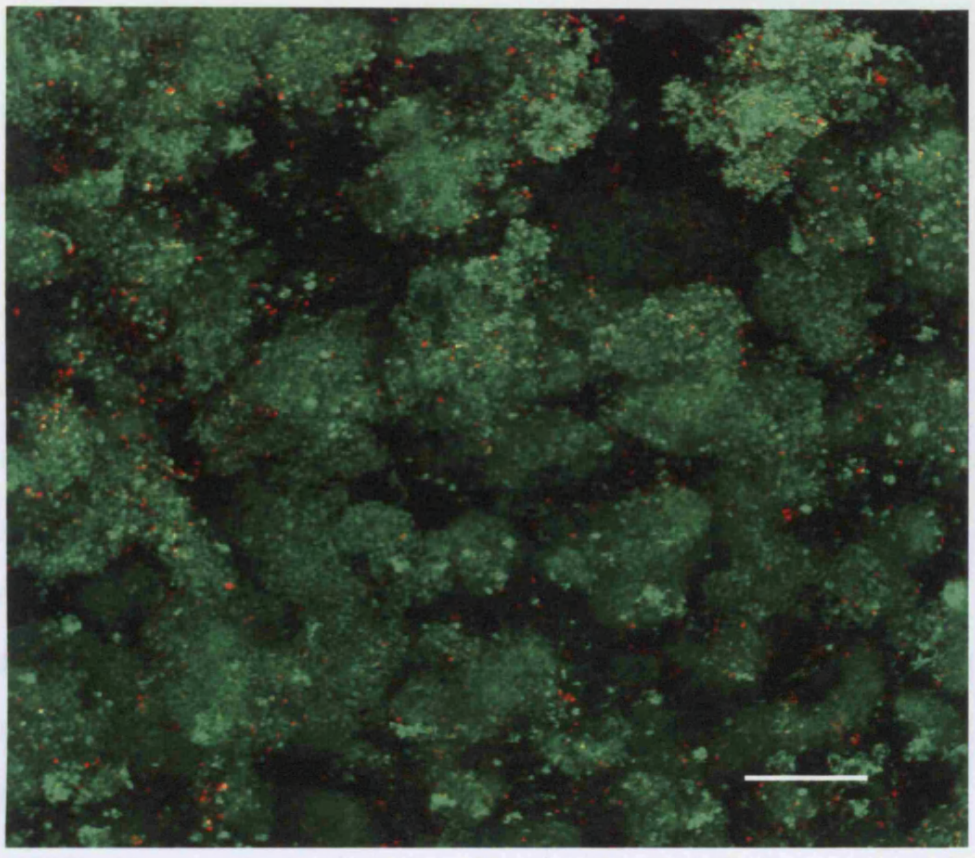


Figure 1.1: A confocal micrograph of a CDFF-cultivated multi-species biofilm, the bar represents 20 μm

The component of bacterial biofilms which is essential for establishment and propagation is EPS, itself a product of bacterial metabolism (Sutherland, 2001). The proportions and physical properties of the extracellular polysaccharides are dependent on a number of factors including the species and relative amounts of bacteria present and the prevailing physical conditions where the nascent biofilm is forming (Sutherland., 2001).

Moreover, the polysaccharides will interact with other compounds present within the localised microenvironment some of which may be of bacterial origin such as enzymes, whilst others may be inorganic, such as charged monovalent and polyvalent ions (Sutherland., 2001).

1.1.2 Biofilm adherence

Microbial deposition is dependent on initial events taking place before accretion and biofilm formation may take place. Any substratum surface that is enveloped in an aqueous environment with compounds present of a biological origin, will rapidly become covered in these organic molecules, referred to as a 'conditioning film' (Schneider et al., 1994). This is then followed by transit of the surrounding microbes to the surface under the influence of various forces such as gravity or Brownian motion (Bos et al.,1999). Upon contact with the conditioning film, the attachment is initially reversible but over a period of time (1-2 minutes) becomes irreversible (Neu et al., 1990). The attachment of the sessile bacterium to the substratum is now dependent on the cohesive strength of the conditioning film (Busscher et al., 1992). Bacterium-substratum and bacterium-bacterium interactions have tended to be defined either in terms of specific molecular interactions which exert an attractive force over a relatively small distance and are highly specific. Or, alternatively, as purely non-specific van der Waals forces or electrostatic interactions, however, in essence, all of the aforementioned interactions originate from the same fundamental forces (Van Oss.,1995).

1.1.2.1 Non-specific interactions

Biofilm adherence may be characterised via the physico-chemical interactions between a bacterium and a substratum (Bos et al., 1999, Busscher et al., 1993). Thermodynamics has been applied to microbial adhesion, in the thermodynamic approach the interacting surfaces are assumed to be in contact with each other under conditions of thermodynamic equilibrium (Absolom et al., 1983). The interfacial free energies are compared between the interacting surfaces (Bos et al., 1999). Hence, this comparison is expressed in the so-called free energy of adhesion (Bos et al., 1999). Alternatively, the classical DLVO (Derjaguin, Landau, Verwey, Overbeek) approach describes the interaction energies between the interacting surfaces, based on Lifshitz-van der Waals and electrostatic interactions and their decay with separation distances (Bos et al.,1999). Both methods prove adequate for characterising some of the adhesion events occurring within the oral cavity, however the heterogeneous nature of the surfaces associated with bacterial adhesion in the oral cavity and the presence of a conditioning film means these approaches are of limited practical use.

1.1.2.2 Specific interactions

Many of the organisms which initially colonise a surface possess an array of cell surface associated appendages which recognise a wide range of organic compounds, protein, glycoprotein or polysaccharide epitopes (Kolenbrander et al.,1993) . All bacteria involved in initial adherence within the oral cavity possess molecules located at the cell surface which facilitate bacterial adherence (Kolenbrander et al., 1993). The earliest colonisers of an enamel

surface have been previously shown to be overwhelmingly streptococci (Nyvad et al., 1987a). It was shown that streptococci accounted for 47-82% of the total cultivable bacteria 4 hours post professional cleaning on enamel surfaces (Nyvad et al., 1987a). A separate study found that the dominant strain in initial streptococal adhesion was *Streptococcus sanguinis* (Bloomquist et al., 1996). Both streptococci and *Actinomyces* species, via specific interactions, will bind to components of the acquired salivary pellicle, much of which is host-derived (Kolenbrander et al., 1993). This initial phase of adherence, which is very much dependent on streptococci in the oral cavity, provides the basis for successive bacterial adhesion events and diversification of community structure which is inherent in dental plaque (Skopek et al., 1993)

1.1.3 Biofilm accumulation

Initial bacterial adhesion events in the oral cavity are rapidly followed by bacterial accumulation, this is a manifestation of the ability of oral bacteria to co-aggregate. Co-aggregation of oral species is a phenomenon that has been extensively catalogued. Initial investigations noted the specificity of co-aggregation, for example the co-aggregation of a *Actionomyces naeslundii* type 1 with two strains of *Streptococcus sanguinis,* but not with any of the other strains of *S. sanguinis* or indeed any other streptococci tested (Gibbons et al., 1970). The investigators concluded that such specificity could only be a result of a stereo-chemical interaction between the two co-aggregating bacteria (Gibbons., 1996). The elucidation of these specific interactions seemed to show that lectin-like structures on the surface of one bacterium

were directly interacting with sugar moieties present on the co-aggregating bacterium. This observation being confirmed by the fact that co-aggregation may be inhibited by lactose and other galactosides (Kolenbrander et al., 1993). The highly specific nature of co-aggregating events between species of streptococci and actinomyces indicated their non-random nature (Kolenbrander et al., 1993). This series of specific co-aggregating events results in the sequential deposition of bacteria as a direct result of their prodigious ability to co-aggregate. Bacterial accumulation also has a definitive spatial distribution whereby the position occupied by a bacterium is a direct result of its capacity to co-aggregate (Demuth et al., 2001, Palmer et al., 2001, Lamont et al., 2002). A bacterium which seems to have a crucial role in biofilm maturation is *Fusobacterium nucleatum* due to its ability to bind to a wide range of bacteria in a specific manner (Kolenbrander et al., 1993), hence allowing a 'late-coloniser' such as *Actinobacillus actinomycetemcomitans* to adhere (as shown in figure 1.2).

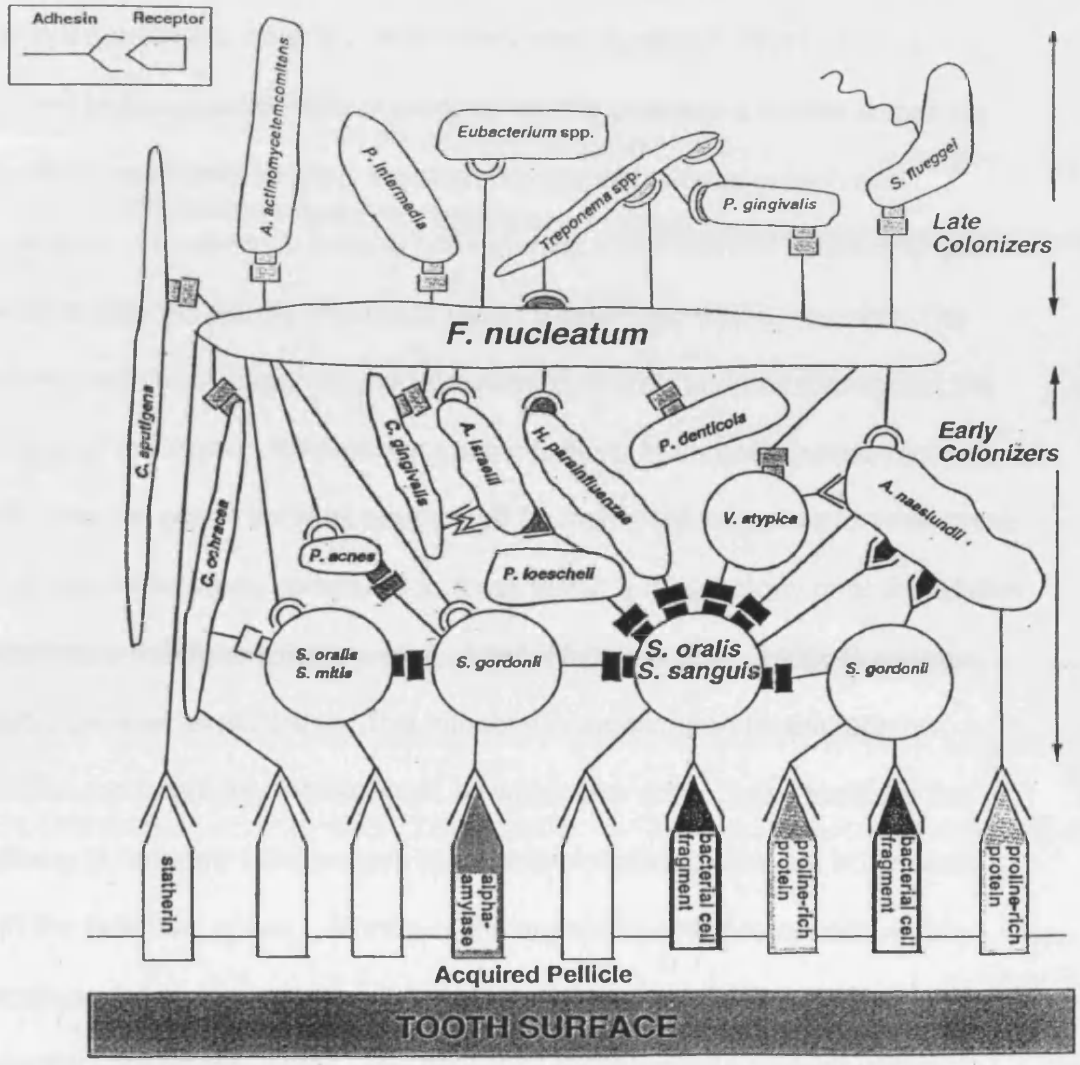


Figure 1.2: Adapted from Kolenbrander et al., 1993, a diagrammatic representation of bacterial aggregation.

1.1.4 Biofilm metabolism

Upon bacterial colonisation and establishment of a biofilm within a new habitat, the nascent biofilm is dependent on that environment for the provision of essential nutrients. However, it is widely postulated that in the environments commonly associated with biofilm formation, such as the oral

cavity, the prevailing nutrient conditions may be such that the bacteria present are in a permanent state of nutrient starvation (Carlsson 1997). Hence, nutrient levels present in the immediate vicinity of where a biofilm is forming are often insufficient to allow the maximal rate of bacterial growth and replication. However, to imply a homogeneity in the nutrient availability and hence metabolic activity of all cells within a biofilm would be incorrect. The biofilm phenotype inherently introduces gradients of nutrients throughout the z-plane of the biofilm. Whereupon concentrations of carbohydrates, protein and essential gases such as oxygen will be higher for the cells proximal to the bulk fluid phase when compared to those within a microcolony or at the biofilm substratum interface (Sissons et al., 1995, Hamilton et al., 1989, Bradshaw., 1995., De Beer et al., 1993). This situation is ameliorated by the fact that biofilms are frequently interspersed by water channels. These facilitate the delivery of nutrients (and oxygen in aerobic biofilms) to cells not in contact with the bulk fluid phase. Moreover the metabolic activities of cells which constitute the biofilm may result in the formation of gradients in pH and redox potential (Bradshaw.,1995). The degree of heterogeneity present within biofilms, certainly multi-species biofilms, has led to their comparison with a multicellular organism (Costerton.,1995). This diversity, and the concomitant gradients, allows the persistence of a wide range of bacteria within the confines of a biofilm each with a particular niche as a result of the metabolic activities of the microbes within the biofilm.

1.1.5 Biofilm nutrition

Many bacterial biofilms formed supra-gingivally in the oral cavity are reliant on sugars as their primary carbohydrate source (see figure 1.3). The state of continual exogenous nutrient flux in the oral cavity with respect to dietary carbon does not present itself as a major problem to the bacteria forming oral biofilms. It is overcome via the utilisation of organic compounds naturally endogenous to that environment coupled with the storage of carbon intracellularly (Poolman, 1993, Frandsen, 1994). Accordingly, many oral streptococci can synthesise compounds resembling glycogen which may then be stored (Marsh et al., 1992). Oral bacteria are adept at the utilisation of glycoproteins which are constituents of saliva (Marsh et al., 1992). This utilisation of glycoproteins demands that bacteria are equipped with an array of appropriate enzymes, including exoglycosidases and glycolytic enzymes to enable degradation (Poolman, 1993). However, no one bacterium possesses the required enzymatic complement to allow complete degradation of these salivary glycoproteins, therefore bacteria must act synergistically to fully degrade these compounds; pure bacterial cultures have previously been shown to poorly metabolise endogenous organic compounds (Homer et al., 1992, Marsh et al., 1992). Oral bacteria are also enzymatically adept at the utilisation of the dietary carbon which sporadically fluxes in the oral cavity. Sucrose occupies a central role in bacterial metabolism supra-gingivally (Marsh, 2003). There are several ways in which sucrose may be metabolised, it may be digested extracellularly with the resultant monosaccharides (fructose and glucose) transported into the cell (Marsh et al., 1992). Sucrose may also be transported into the cell as a disaccharide or disaccharide phosphate and

digested intracellularly via invertase or sucrose phosphate hydrolase (Marsh et al., 1992). Finally, sucrose may be utilized extracellularly by glucosyltransferases . Glucosyltransferases produce soluble and insoluble glucans which are important in plaque formation. Fructosyltransferases produce fructans which are frequently labile and can be used by other plaque organisms (Marsh et al., 1992). Hence, biofilm bacteria within the oral cavity, certainly those supra-gingivally, must display a high degree of adaptability, utilising either the carbon components of salivary glycoproteins or the transient dietary carbon.

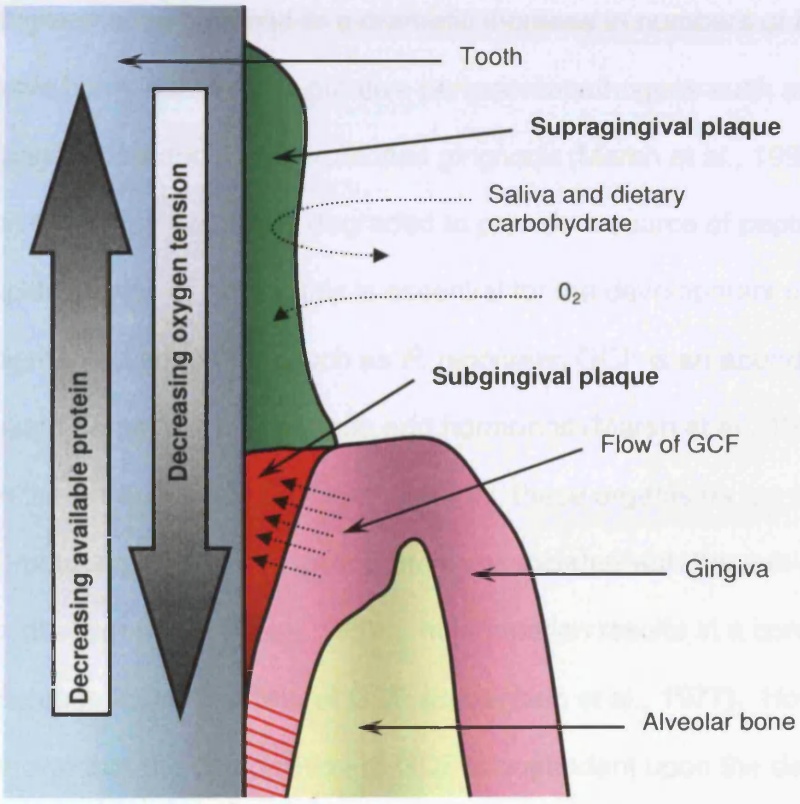


Figure 1.3: Gross structure of the gingival crevice showing the location of supra and sub-gingival plaque (courtesy of Dr Christopher Hope of the Eastman Dental Institute).

The situation for sub-gingival biofilms is somewhat different. Many of the bacteria found sub-gingivally and within periodontal pockets are asaccharolytic but proteolytic (Marsh et al., 1992, Carlsson 1997). The bacteria commonly found in this environment utilise the protein content of gingival crevicular fluid (GCF) for growth (Marsh et al., 1992) (see figure 1.3). When inflamed (inflammation is a direct result of bacterial colonisation and biofilm formation), many more host-derived nutrients are presented to the microbiota. GCF is known to harbour components that are commonly found in serum including; immunoglobulins, components of the complement cascade, fibrinogen, transferrin and albumin (Estreicher et al., 1996). This nutrient augmentation can lead to a dramatic increase in numbers of bacteria which have been identified as putative periodontopathogens such as *Tannerella forsythensis* and *Porphyromonas gingivalis* (Marsh et al., 1992). These proteins may be rapidly degraded to provide a source of peptides and amino acids as well as haem, this is essential for the development of black-pigmented anaerobes such as *P. gingivalis,* GCF is an abundant source of haem-containing compounds and hormones (Marsh et al., 1992). In addition to the breakdown of GCF components, these organisms are able to degrade structural proteins and glycoproteins associated with the sub-gingival epithelium (Marsh et al., 1992). Inflammation results in a concomitant increase in the flow rate of GCF (Schenkein et al., 1977). However, it is known that the composition of GCF is dependent upon the degree of gingival inflammation. It has been reported that the average total protein content of GCF from 'healthy' non-inflamed sites was c.22 mg/ml, at inflamed sites this increased to range between 69-74 mg/ml (Curtis et al., 1988). Indeed a

separate analysis of the protein concentration of GCF at inflamed sites reported a concentration of 70 mg/ml (Bickel et al., 1985), consistent with what was found in the previous study.

1.1.6 Biofilm resistance to antimicrobials

One of the defining characteristics of biofilms is their inherent recalcitrance to antimicrobial agents. This characteristic is highlighted by work on a biofilm formed from an adherence-deficient strain of *Staphylococcus epidermidis* when compared to nascent wild-type biofilm. Unsurprisingly, the adherence-deficient phenotype was much more effectively eradicated than the wild type (Schwank et al., 1998). This exemplifies the statistic that bacterial cells within a biofilm become approximately 10 – 1000 times less susceptible to the effect of any applied antimicrobial agent (La Tourette Prosser et al., 1987, Mah et al., 2001). The significance of this observation becomes clear when it is considered that biofilms have been implicated in 65% of nosocomial infections, the resultant treatment of these infections costs approximately $1 billion annually in the USA (Archibald et al.,1997). A plethora of experimental work has demonstrated several mechanisms via which biofilms may survive the application of antimicrobial agents. These include an inherent resistance to the applied antimicrobial (Nikaido, 1988), this may be a function of the physiological conditions prevailing in and around the biofilm or due to the expression of a novel phenotype hitherto not seen in the planktonic state (Gilbert et al., 1997). It has been suggested that resistance may be due to the acquisition of a mobile element or a genetic mutation leading to the emergence of a resistant phenotype (Gilbert et al., 1997). Undoubtedly

biofilm resistance may be understood to be a chimera of all of these mechanisms resulting in the resistance phenomenon that is characteristic of biofilms.

1.1.6.1 Biofilm resistance to due to decreased growth rate

It has been hypothesised that decreased growth rate may contribute to biofilm recalcitrance to antimicrobial agents (Brown et al., 1988). It is generally understood that all antimicrobial agents are much more effective when utilised against rapidly growing cells (Lewis., 2001). Bacteria growing within a well-agitated steady state system will not encounter significant nutrient or associated physiological gradients and hence a uniformity of phenotype will prevail. Whereby on application of an antimicrobial, a singular response may be expected (Gilbert et al., 1997). The very nature and architecture of biofilms results in the bacteria enclosed within the exopolysaccharide to be exposed to a wide range of gradients in nutrients, oxygen and metabolic by-products. Therefore, within the biofilm, growth rates will be generally suppressed relative to planktonic cells (Gilbert et al., 1997). These factors contribute to a high phenotypic diversity and concomitant variation in antimicrobial susceptibility (Gilbert et al., 1997). Indeed, antibiotics such as penicillin and ampicillin have an absolute requirement for the target cells to be growing if they are to be effective (Lewis et al., 2001).

1.1.6.2 Biofilm resistance due to restricted penetration

Upon growth as a sessile community and the initiation of biofilm formation, a diverse and heterogeneous array of exopolysaccharides are synthesised.

This exopolysaccharide production has multiple roles and aids interbacterial aggregation and the maintenance of biofilm coherence. This exopolysaccharide also presents a physical barrier, impeding antibacterial penetration of the biofilm (Mah et al., 2001). The exopolysaccharide may inhibit the penetration of the applied antimicrobial via ionic interactions and molecular sieving events, thus it may be seen as being analogous to the peptidoglycan layer (Marquis., 1968). The exopolysaccharide may impede antibacterial penetration by acting as a quasi ion exchange column whereby the applied compound must first saturate all available binding sites before it may act on the target organism (Wagman et al., 1975). The glycocalyx also prevents access of phagocytic cells such as polymorphs and macrophages, part of cell-mediated immunity, preventing destruction of the biofilm cells. However, what is evident is that the exopolysaccharide does not present a diffusional barrier to all applied antimicrobials. Experimental studies have demonstrated that the antibiotic ciprofloxacin fully penetrated monospecies biofilms of *Klebsiella pneumoniae* within twenty minutes of application, however this did not produce the same antibacterial effect as had been observed in planktonic cells, the biofilm phenotype conferred resistance (Anderl et al.,2000). Evidently, reduced penetration is not sufficient to explain all instances of this resistance phenomenon.

1.1.6.3 Expression of a biofilm-specific phenotype

Biofilm strategies for decreased susceptibility to applied antimicrobial agents have thus far concentrated on the physical parameters of the biofilm hindering access of the antimicrobial to the target organism or the relative dormancy

seen in biofilm cells due to nutrient deficiency and concomitant slow growth. But, it has been hypothesised that the cells upon forming a biofilm undergo a distinct phenotypic change exhibiting a so-called biofilm specific phenotype (Gilbert et al., 1997, Cochran et al., 2000, Mah et al., 2001). This expressed phenotype may not be exhibited by all of the cells constituting the biofilm, however a certain sub-set would undergo this phenotypic shift making them better able to resist the effects of antimicrobial application (Mah et al., 2001). A pertinent example of this is the seeming link within *Escherichia coli* cells between growth rate and expression of the *mar* operon that confers multiple antibacterial resistance. It seems that there is an inverse relationship between expression of the *mar* operon and growth rate, so as doubling time slows so expression of this multiple resistance operon increases (Maira-Litran et al., 2000). This correlates with what is hypothesised to be the nutrient sparse conditions, and hence reduced growth rate, within a microcolony leading to possible upregulation of regulons such as *mar.* However, for many studies there has been no positive correlation between growth as a biofilm and the induction of any overtly biofilm-specific phenotype that would explain the multiple instances of biofilm resistance to antimicrobial application.

1.1.6.4 The effect of 'persistor' cells

The resistance mechanisms examined thus far are applicable to every cell which constitutes a biofilm, or at least applicable to a sizeable part of the population such as cells deep within a microcolony (Costerton et al., 1999b,). However, these generalised resistance mechanisms may not be the primary way in which biofilms persist in conditions that are unconducive for planktonic

cells. As detailed previously, the biofilm phenotype does not present itself as a barrier to all applied antimicrobials, the example cited being the antibiotic ciprofloxacin. It has been shown that the fluoroquinolones equilibrate throughout a stratified biofilm. Fluoroquinolones have also been shown to be bactericidal against non-growing planktonic cells when suspended in phosphate buffer. This result contrasted with what was found using biofilm cells, which when resupended in media were more resistant to the antibiotic than the non-growing planktonic cells (Brooun et al., 2000). Furthermore the fluoroquinolones ciprofloxacin and ofloxacin upon application to *Pseudomonas aeruginosa* biofilms at clinically achievable concentrations resulted in up to a four log reduction in the viable count. However upon incremental increase in the concentration of antibiotic applied, the viable count was not reduced any further, the surviving cells within the biofilm being termed 'persistor' cells due to their precocious ability to withstand antimicrobials (Brooun et al., 2000).

The 'persistor' cells, which numerically form a tiny proportion of the biofilm population, seem to be implicated in the ability of biofilms to withstand, and recover from, the effect of antimicrobial application (Lewis., 2001). It is highly unlikely that this phenomenon is limited to biofilm cells; however planktonic cells which are not eliminated by the action of an antimicrobial are rapidly dealt with by the immune system which clears the remaining cells. The 'persistor' cells within a biofilm benefit from the relatively remote conditions produced within a biofilm. The biofilm itself being a barrier to the immune system (Lewis., 2001). Hence, the concerted action of the applied

antimicrobial and the immune system prevents the recurrence of many planktonic microbial infections, this synergy proves impossible when dealing with infections due to the bacterial biofilms (Lewis., 2001). One may view the biofilm survival mechanism as merely a variant of that utilised by planktonic cells, the notable difference being the absence of a tangible immune response (Lewis., 2001). Therefore, the question remains as to what a persistor cell actually is. Joseph W. Bigger, in work carried out in 1944 on the action of penicillin on "*Staphylococcus pyogenes*", noted what he termed as the 'persistor' phenomenon, and established that upon further re-growth, persistor cells reverted to the wild-type phenotype (Bigger., 1944). Certain facts as to the nature of persistors have already been established, what is clear is that persistors are not mutant cells nor do they represent a special, or hitherto unobserved, stage in the cell cycle (Lewis., 2001). Nor are persistor cells in a dormant state. It is yet to be established, however, what environmental conditions e.g. nutrient availability, are conducive to the emergence of persistor cells, or whether persistor cells themselves may be broken up into further sub-populations depending on varying resistance to antimicrobials (Lewis., 2001). Persistors are an interesting group of cells and may go some way to explaining the recalcitrance of biofilm-bound cells to applied antimicrobials.

1.2 Oral Biofilms

Biofilms within the oral cavity are highly variable, with microbial composition varying between sites. This microbial diversity is a function of the highly protean micro-environments that are to be found within the oral cavity.

However, a broad distinction may be made between biofilms that form in the oral cavity either supra- or sub-gingivally.

Supra-gingival biofilms will be subjected to a high shear environment due to mastication, chewing and ingestion of food. Biofilms forming here will be in an aerobic environment, the main energy source being carbohydrates which are endogenous, with sporadic exogenous supplementation primarily in the form of sucrose. The supra-gingival biofilms or dental plaques are highly diverse with up to 500 taxa being present (Rosan et al., 2000). Initially supra-gingival biofilms are dominated by Gram-positives; if left to develop undisturbed, the biofilm will diversify to include large numbers of facultative and obligately anaerobic Gram-negative organisms (Loe et al., 1965). The composition of supra-gingival biofilms has been a subject of vigorous scientific scrutiny, such that we now have a comprehensive understanding of supra-gingival plaque formation. Culture-based studies have been performed on the successional events that occur concomitantly with plaque maturation; initially the pioneer species are predominatly streptococci with Gram-positive rods such as the *Actinomyces* constituting an appreciable fraction of the total cultivable flora (Nyvad et al., 1987a, Zee et al., 1996). As the plaque matures, Gram-positive rods form a larger fraction of the total cultivable flora, indeed Gram-negative rod species become much more prevalent (Nyvad et al., 1987a, Zee et al., 1996). The dynamic nature of supra-gingival plaque formation is graphically demonstrated if one compares the most frequently isolated organisms on day 1 of the Zee et al study when compared to day 14, day 1 marked the

beginning of a period where the cohort abstained from oral hygiene. Hence on day 1, *Staphylococcus epdermidis*, *Veillonella dispar*, *Actinomyces israelii*, *Propionibacterium granulosum*, *Gemella morbillorum*, *Streptococcus mitis* and *Streptococcus sanguinis* were the most frequently isolated; by day 14 the microbiota had shifted to include appreciable numbers of *Prevotella intermedia*, *Fusobacterium* and *Capnocytophaga* species (Zee et al., 1996). Indeed, plaque formation in occlusal fissures follows a somewhat similar pattern, with the vast majority of cultivable microbiota being Gram-positive cocci and rods, with *streptococci* and *Actinomyces* species to the fore (Theilade et al., 1982). In contrast to the previous studies, however, Gram-negative rods were only infrequently isolated with Gram-negative cocci being more numerous (Theilade et al., 1982). Recently, culture-based analysis has been supplemented with molecular techniques; one such methodology is the checkerboard DNA-DNA hybridization technique. This technique was employed to monitor bacterial colonisation during *de novo* plaque formation; prior to sampling inter-proximal sites a preparatory phase was necessary, this involved meticulous, professionally-administered, oral hygiene (Ramberg et al., 2003). At the commencement of the study, the supra-gingival microbiota accreting to form nascent biofilms was dominated by *Actinomyces* species, these organisms comprised c.50 % of the microbiota evaluated. Over the four successive days of sampling, it was found that four *Streptococcus* species; *S. gordonii*, *S. oralis*, *S. sanguinis* and *S. anginosus* increased appreciably as a percentage of the microbiota, whilst numbers of the genus *Actinomyces* remained constant (Ramberg et al., 2003). Moreover, *A. actinomycetemecomitans*, *Capnocytophaga gingivalis*, *Capnocytophaga*

ochracea, *Capnocytophaga sputigena*, *Eikenella corrodens*, *Campylobacter rectus*, *Capnocytophaga showae* and *F. nucleatum* also increased in number appreciably over the four days of sampling (Ramberg et al., 2003). Interestingly, although the putative periodontal pathogens *T. forsythensis*, *P. gingivalis* and *Treponema denticola* were detected, they formed a numerically tiny proportion of the microbiota (Ramberg et al., 2003). Hence, one may conclude that supra-gingival plaque is dominated by Gram-positive species, most notably streptococci and *Actinomyces* spp., with low proportions of other species including putative periodontal pathogens present (Ramberg et al., 2003).

The situation sub-gingivally, however, is very different as the environment may be anoxic with a low Eh, there are no significant shear forces, and the bacteria present derive their nutrients from GCF. GCF has a high protein content, and the prevailing conditions allow the colonisation of bacteria which are generally proteolytic. Sub-gingival plaque appears under the light microscope as a mass of bacterial cells with filamentous bacteria at right angles to the pellicle, sub-gingival plaque may also have a loosely-arranged zone of motile organisms (Saxton, 1973). The bacterial diversity sub-gingivally has been exhaustively analysed, however much of this research has focused on the bacteriology of periodontal diseases, the number of phylotypes associated with periodontal health is perhaps not as wide-ranging as may be isolated from a disease lesion. The subgingival microbiota associated with periodontal health was examined by Tanner *et al.*, with the cultivable bacteria being identified. The dominant cultural microbiota was composed of similar

mean proportions of Gram-positive rods and cocci, principally *Actinomyces naeslundii*, *A. genercseriae*, *S. oralis* and *Micromonas micros* (Tanner et al., 1998). In another study, the most numerous species isolated from periodontal crevices of subjects in periodontal health were catalogued; *A. naslundii*, *S. sanguinis*, *V. dispar*, *F. nucleatum*, *S. oralis* and *S. intermedius* were the most numerous cultivable species (Moore and Moore., 1994). Interestingly, Gram-positive species are well represented, in concurrence with what was found by Tanner at al., (1998). The sub-gingival bacterial diversity has also been investigated using culture-independent molecular methods. A methodology that does not depend upon an initial culture stage allows the investigator to detect species that may well reside in numbers below the detection limit of a cultural assay as well as those species that cannot yet be cultured in the laboratory. Using a culture-independent technique, 72 species or novel phylotypes have been identified that are associated with periodontal health (Paster et al., 2001). The species most commonly associated or recovered from healthy subjects included *Streptococcus* spp. and *Corynebacterium matruchotti*; species or phylotypes commonly detected in both health and disease include *Neisseria mucosa*, *Campylobacter gracilis*, *Campylobacter concisus*, *T. denticola*, *Fusobacterium* spp., *Leptotrichia buccalis*, *Actinomyces naeslundii* II and *Rothia dentocariosa* (Paster et al., 2001). Indeed, as with the supra-gingival milieu, the microbiota indicative of periodontal health seems to be composed principally of Gram-positive species.

1.2.1 Diseases due to oral biofilms

Dental plaque is an integral part of the oral environment, the interactions between host and the various bacteria are regarded primarily as symbiotic with the plaque bacteria occupying all available niches preventing the colonisation of any exogenous species which may be potentially pathogenic. However, this delicate equilibrium may be easily upset if dental plaque is able to proliferate without frequent mechanical debridement. The oral diseases that are prevalent throughout the world are a manifestation of unchecked dental plaque proliferation, these diseases being dental caries and periodontal diseases. The importance of oral biofilms and certain endogenous opportunistic pathogens has been established in the aetiology of oral diseases.

1.2.2 The specific versus non-specific plaque hypotheses

Two main hypotheses as to the precise role of oral biofilms in diseases such as caries and periodontal diseases were developed as research into oral diseases intensified. The specific plaque hypothesis states that whilst bacterial biofilms are diverse and heterogeneous with a high degree of species diversity evident, oral diseases are a result of the action of a limited and defined sub-set of microbes resident in oral biofilms (Loesche, 1976). Hence, when attempting to prevent and treat oral diseases, the most effective way to accomplish this is to eradicate the putative pathogens (Marsh.,1999). This approach is typified by research carried out by Bass and Johns in 1915, here they identified a specific organism as the agent responsible for periodontal disease - *Endomeba buccalis.* The recommended remedial action

was to vaccinate against this particular organism in the hope of preventing periodontal disease (Christersson et al., 1991). The alternative explanation is known as the non-specific plaque hypothesis. It expounds that oral disease is the inevitable consequence of the unchecked activities of the microorganisms which constitute oral biofilms. This hypothesis considers that oral disease is a poly-microbial infection and that no one microbe may be defined as a pathogen, but disease is the net result of the overall plaque activity (Theilade, 1986).

However one must consider the aetiology of oral diseases, there is a wealth of circumstantial evidence demonstrating a modicum of specificity, whereby a limited number of bacteria are consistently isolated from disease lesions (Marsh, 2003). An alternative hypothesis has been proposed which includes elements of both hypotheses producing a more complete analysis as to the role of biofilms in oral disease. This hypothesis, known as the ecological plaque hypothesis (figure 1.4), states that putative pathogens may be ubiquitous in the oral environment, however in areas which are clinically healthy their numbers are too low to result in the onset of disease (Marsh.,1994). Disease is initiated due to changes in the local micro-environment, these result in an ecological shift of the resident microflora whereupon relative numbers of the pathogenic organisms increase bringing about the onset of disease. Hence, the key features of this hypothesis expound that increases in a putatively pathogenic flora are a direct consequence of physiological changes occurring in the oral cavity, producing conditions within which this flora may flourish (Marsh, 2003). Additionally, oral

diseases do not have a specific aetiology, hence any bacterial species possessing the relevant traits may contribute to the disease process (Marsh, 2003); the results obtained by Moore et al., 1982 exemplify this, they examined the bacteriology of severe periodontitis in young adults, they found that the cultivable floras of the individuals sampled significantly differed from each other.

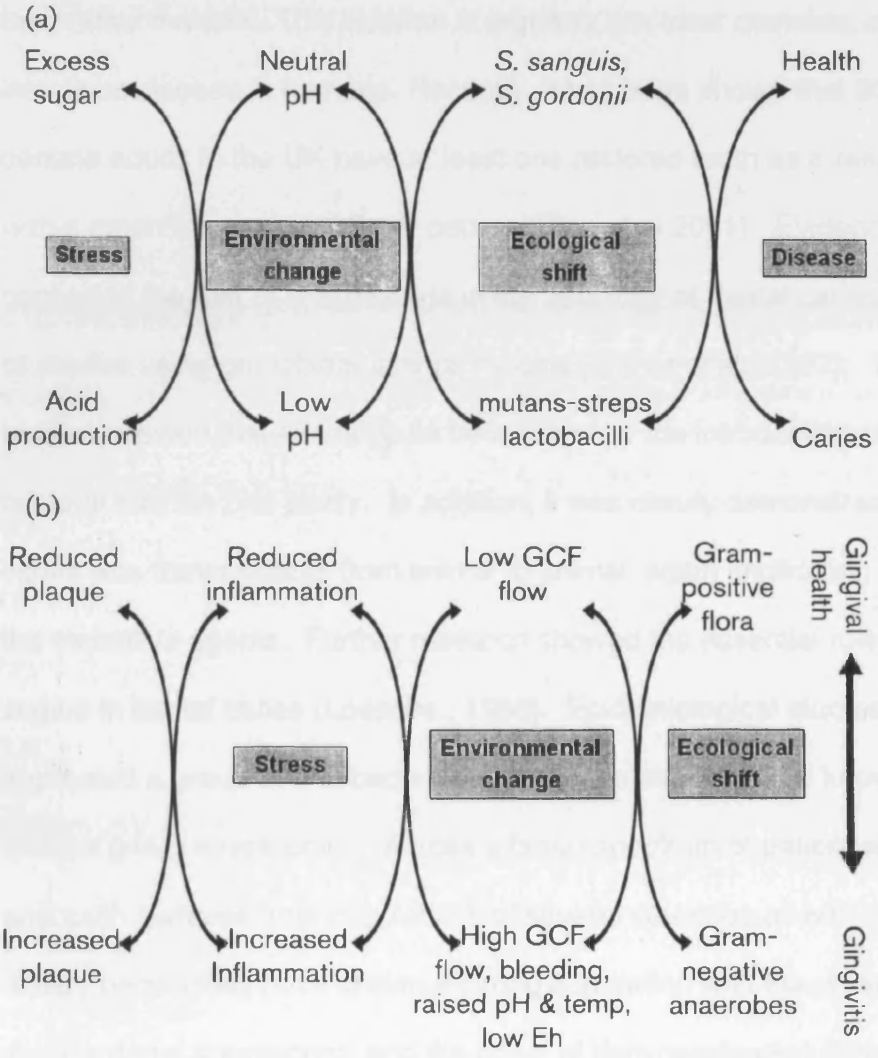


Figure 1.4: Adapted from Marsh, 1994, the ecological plaque hypothesis demonstrates the effect of environmental shifts with concomitant perturbation of the resident microflora and how this results in (a) caries and (b) the onset of gingivitis.

1.2.3 Dental plaque and caries

In industrialised society dental caries is one of the most prevalent diseases affecting the general populace (Marsh.,1999). Dental caries can be defined as the localised demineralisation of the tooth tissue by various acids produced by bacterial fermentation of dietary carbohydrates. Treatment of the disease involves removal of the damaged tooth tissue and its replacement with a restorative material. This disease is arguably the most common, chronic infectious disease in humans. Recently, it has been shown that 90% of all dentate adults in the UK have at least one restored tooth as a result of caries with a mean frequency of 7 per person (Pine et al 2001). Evidence which confirmed the role of oral bacteria in the aetiology of dental caries was a result of studies using gnotobiotic animal models (Zinner et al., 1967). These studies showed that caries could be induced by the introduction of specific bacteria into the oral cavity. In addition, it was clearly demonstrated that caries was transmissible from animal to animal, again implicating bacteria as the causative agents. Further research showed the essential role of dietary sugars in dental caries (Loesche., 1986). Epidemiological studies have implicated a group of oral bacteria in dental caries, these are known as the mutans group streptococci. Across a broad spectrum of patient age range and tooth surfaces from populations of several countries all with unique dietary pecularities have shown a strong correlation with the presence of mutans group streptococci and the onset of demineralisation (Loesche., 1986). The progression of dental caries is dependent on the concerted efforts of many plaque bacteria commonly isolated from mature dental plaques. Therefore the microbiota may contain species which are sufficiently

acidogenic to demineralize cementum and dentine whilst proteolytic organisms would degrade the dentine collagen matrix (Marsh.,1999). Hence, the disease process is continued by the continual bacterial succession from disease onset through to the production of a lesion. As the lesion progresses through the dental tissue, the microbiota alters in response to changing environmental conditions to include a higher percentage of facultative and obligately anaerobic species (Marsh.,1999). There are several pathogenic determinants of cariogenic bacteria which enable demineralization and hence the production of a carious lesion. Cariogenic bacteria are adept at the transport of fermentable sugars and their subsequent metabolic conversion to acid. Cariogenic bacteria also produce copious amounts of both extra and intracellular polysaccharides, and they maintain sugar anabolism and catabolism at an acidic environmental pH which is actually conducive to their growth (Marsh.,1999).

1.2.4 The structure of the periodontium

The 'periodontium' is a term used to designate the functional structures which are directly involved in resisting forces applied to the teeth. These structures are the gingiva, periodontal ligament, cementum and alveolar bone (Jenkins and Allan, 1994) (see figure 1.5 for a diagrammatic representation). The gingiva is the fibrous mucosa surrounding the teeth and covering the coronal portion of the alveolar process; it is pink in colouration in contrast to the red of the alveolar mucosa and is lined by a keratinised stratified squamous epithelium (Jenkins and Allan, 1994). The periodontal ligament attaches the tooth to the alveolar bone, fibroblasts are the principal cell line, however the

main component of the periodontal ligament is collagen. The primary function of the periodontal ligament is to dissipate load and support the tooth during mastication (Jenkins and Allan, 1994). The alveolar bone surrounds the roots of erupted teeth, stopping just before the amelocemental junction. Indeed, the entire root is covered in a thin layer of cementum. At the amelocemental junction, the cementum either abuts or overlaps the enamel, occasionally it will stop short of it leaving exposed dentine. Cementum is similar in composition to bone but is avascular and not innervated (Jenkins and Allan, 1994).

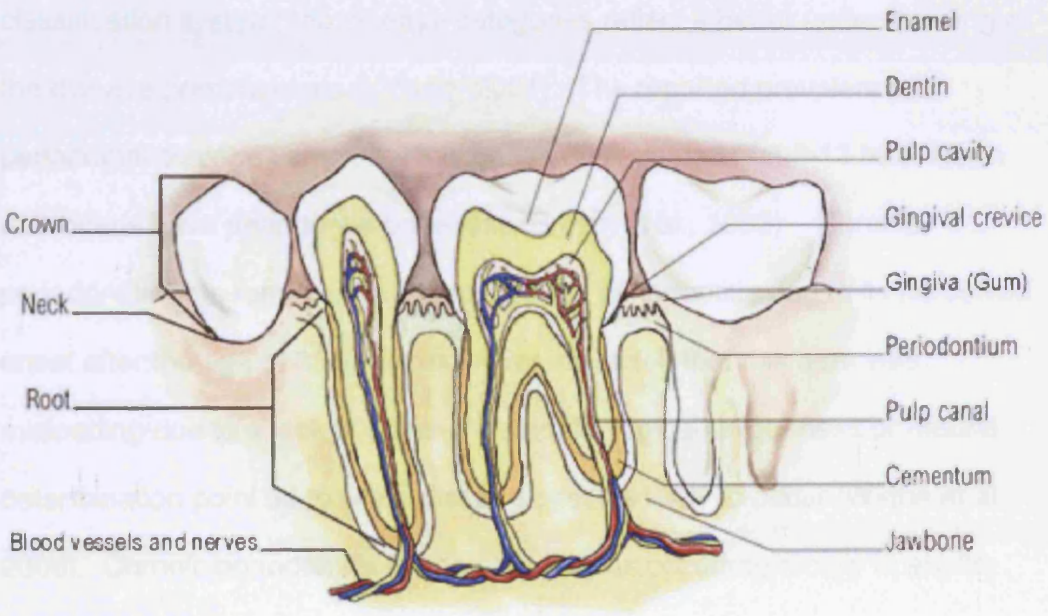


Figure 1.5: Adapted from http://www.kindandental.com/images/atlas-tooth.jpg a cross-section of a tooth and surrounding structures.

1.2.5 Periodontitis

The formation and proliferation of bacterial plaques sub-gingivally are the causative agents of periodontitis (Socransky, 1970). Periodontitis is one of

the most common forms of chronic disease affecting the western world. The term periodontitis is used to demarcate a group of inflammatory diseases which affect all periodontal structures; the periodontium including gingiva, gingival attachment, periodontal ligament, cementum and supporting alveolar bone (Meghji et al., 1992). Periodontitis results from the apical extension of a gingival inflammatory process. Destruction of the periodontal attachment results in the formation of a periodontal pocket (Jenkins and Allan, 1994).

Periodontal diseases have recently been the subject of a re-classification, such that the current system is somewhat simpler than the previous classification system; the disease categories reflect a better understanding of the disease presentations (Kinane, 2001). The reported prevalence of periodontal disease varies, but it is generally accepted that 8-13 % of North Americans have periodontal bone loss (Brown et al., 1993). Chronic periodontitis was formerly classified as adult periodontitis due to its perceived onset after the age of 35 years; however it was felt that this term was misleading due to a lack of either a histopathological uniqueness or natural determination point as to when disease onset is likely to occur (Wiebe et al., 2000). Chronic periodontitis is characterised as occurring mostly in adults. Destruction is consistent with the quantity of plaque present; sub-gingival calcified plaque (calculus) is also found. In general, the disease progresses slowly, interspersed with bursts of destruction (Wiebe et al., 2000). Chronic periodontitis may be designated as localised or generalised, dependent upon whether less than, or greater than, 30 % of sites within the mouth are affected (Kinane, 2001). There are forms of periodontal disease that clearly differ from

the parameters established for chronic periodontitis. Aggressive periodontitis is characterised by rapid attachment loss and bone destruction with possible familial aggregation of disease (Wiebe et al., 2000). Aggressive periodontitis can be considered either localised or generalised as was the case with chronic periodontitis. The term 'incidental attachment loss' is now used to denote a form of periodontitis that is localised in nature but cannot easily be categorised as either chronic or aggressive periodontitis (Kinane, 2001). It is well known that systemic diseases that affect immune function, inflammatory response and tissue organisation can modify the onset and progression of all forms of periodontal disease. Hence periodontitis associated with a systemic disorder is classified as 'periodontitis as a manifestation of systemic diseases' (Wiebe et al., 2000). Necrotizing periodontal diseases now encompass two former disease categories, necrotizing ulcerative gingivitis and necrotizing ulcerative periodontitis. Both diseases appear to be related to diminished systemic resistance to bacterial infection, and differ in terms of the tissue affected, thus the disease may be limited to the gingiva or may have progressed to also involve the attachment apparatus (Wiebe et al., 2000).

1.2.6 Role of bacteria in periodontitis

The microbiota associated with periodontitis has been subjected to intense scientific scrutiny over the past half century; this endeavour has led to an ever growing understanding of the flora which one may recover from a periodontal lesion *in vivo*. It is now known that the complexity of the human periodontal flora rivals that of the gastrointestinal tract (Moore and Moore, 1994). Furthermore, the abundance and diversity of bacterial species in the

periodontal flora and vacillation in the precise composition of floras from individual to individual and variation in the host response to bacterial species means that it is difficult to define a specific disease aetiology (Moore and Moore, 1994).

For the initiation of the disease process, certain criteria must be fulfilled. The host must be susceptible to disease and the putative pathogens present. The environmental conditions must be conducive and the number of pathogenic species present must exceed the threshold tolerable for that site (Socransky et al., 1993). The equilibrium may be upset, the result being a poly-microbial infection which is essentially opportunistic (Van Winkelhoff et al.,1996).

The human-sub-gingival flora has been investigated using a number of differing methodologies including culture, DNA-DNA hybridisation techniques and ribosomal 16 S cloning and sequencing. Moore et al., (1982) examined 78 supra-gingival samples and 42 sub-gingival samples from 21 patients presenting with 'severe periodontitis'. The cultivable taxa which were more numerous supra-gingivally than subgingivally included; 5 species of *Actinomyces*, 3 species of *Leptotrichia*, 10 species of *Streptococcus*, 3 species of *Veillonella*, 3 species of *Staphylococcus*, *C. ochracea*, *Propionibacterium acnes* and *Peptostreptococcus anaerobius.* 11 species were found to exceed 1 % of the total sub-gingival flora and were most closely associated with diseased sulci, this included; 5 species of *Eubacterium*, 2 species of *Lactobacillus*, 1 species of *Bacteroides*, *F. nucleatum*, *S. anginosus* and *M. micros.* Indeed *F. nucleatum* was the most commonly

isolated species from diseased sulci. Moore et al., (1983) examined 22 supra-gingival and 38 sub-gingival plaque samples. A total of 171 taxa from 1900 bacterial isolates were cultured from 22 subjects receiving treatment for 'moderate' periodontitis. The study found that the taxa which predominated in supra-gingival plaque included 4 species of *Streptococcus*, 9 species of *Actinomyces*, 3 species of *Capnocytophaga*, *Veillonella parvula*, *Leptotrichia buccalis*, 2 species of *Selenomonas* and *Rothia dentocariosa*. However, this contrasted with what was found sub-gingivally; 3 *Fusobacterium* species, 2 *Peptostreptococcus* species, 7 *Eubacterium* species, *Campylobacter rectus*, *P. gingivalis* and several *Prevotella* species were found to be the principal taxa. The sub- and supra-gingival microbiota of subjects presenting with chronic periodontitis has also been analysed and contrasted using whole genomic DNA probes and checkerboard DNA-DNA hybridisation techniques (Ximenez-Fyvie et al., 2000). A total of 1170 samples of sub- and supra-gingival plaque samples were collected and evaluated for the presence and levels of 40 bacterial taxa. All 40 of the taxa were detected in both the supra- and sub-gingival plaque. *Actinomyces* species were the most prevalent taxa in both habitats. 75-100 % of the supra- and 62-100 % of sub-gingival sites were colonised by at least one of 5 *Actinomyces* species. Supra-gingival samples exhibited significantly higher counts of *A. naeslundii* genospecies 1, *A. israelii*, *A. odontolyticus*, *Neisseria mucosa*, *S. gordonii*, *C. ochracea* and *C. sputigena* when compared to counts in sub-gingival samples taken from the same tooth surface. In contrast, sub-gingival plaque samples presented significantly higher counts of *Prev. nigrescens*, *Prev. intermedia, T. forsythensis* and *P. gingvalis*, interestingly putative periodontal pathogens

were detected in supra-gingival plaque from sites where sub-gingival samples were negative for the same species; thus giving credence to the postulate that the supra-gingival milieu may act as a reservoir of such species for the dissemination or re-infection of sub-gingival sites (Ximenez-Fyvie et al., 2000). Cloning and sequencing techniques have also been employed to determine the sub-gingival microflora that may be recovered from a periodontal lesion; such culture-independent techniques were employed by Paster et al., (2001) to determine the microbiota associated with both sub-gingival health and disease. This study estimated that the total number of taxa that may be recovered from sub-gingival plaque is c. 415. Indeed they went on to hypothesise that the total species diversity in the oral cavity is c. 500 species, a figure that had been mooted previously (Paster et al., 2001). The species/phylotypes which were most closely associated with periodontitis included; 2 species of *Atopobium, Fusobacterium naviforme, Fusobacterium animalis, Streptococcus constellatus, Gemella haemolysans, Eubacterium saphenum, Filifactor alocis, Catonella morbi, Selenomonas spitigena, Haemophilus parainfluenzae, Campylobacter rectus, Porphyromonas endodontalis, Prevotella tannerae, P. gingivalis,* and *T. forsythensis.* The results of the studies summarised differ in the species which have been recovered from periodontitis lesions *in vivo*; however, as stated previously, although it is rather difficult to define a specific disease aetiology for periodontitis, one may instead describe a trend that is evident when comparing periodontal health to the microbiota associated with periodontitis. Hence, one can conclude that the species indicative of periodontal health are predominantly Gram-positive species, including streptococci and *Actinomyces*

spp., whereas periodontitis is associated with ever larger increases in the proportions of Gram-negative organisms which may be recovered from the sub-gingival milieu; indeed, it is striking how the shift from periodontal health through to periodontitis correlates with an increase in species diversity sub-gingivally.

P. gingivalis has received a great deal of attention as a possible mediator of periodontal destruction; it occurs as a predominant species in the sub-gingival microbiota of some individuals with advanced and severe periodontitis (Moore and Moore, 1994). The presence of this particular organism acting alone or in concert as part of a poly-microbial infection appears to be essential for disease activity (Socransky and Haffajee, 1992). *P. gingivalis* does not elaborate a single exotoxin but a surfeit of enzymes along with toxic metabolites, and cellular constituents which may be produced by the cell (Lamont and Jenkinson, 1998). These compounds have the potential to impinge upon host tissue resulting in alveolar bone loss and the breakdown of other periodontal tissues. In addition, both the innate and acquired immune defence mechanisms may be affected, enhancing the microbially-driven degradation of the periodontium (Lamont and Jenkinson, 1998). Specific examples of tissue degradation and attenuation of host defence mechanisms include the degradation of extracellular matrix proteins, activation of matrix metallo-proteases (MMPs), inactivation of plasma protease inhibitors, cleavage of cell surface receptors, activation or inactivation of complement factors and cytokines, and activation of the kallikreinkinin cascade (Travis et al., 1995). In addition to its potent proteolytic activity, *P. gingivalis* can

degrade the glycosaminoglycans, hyaluronate, chondroitin sulphate and heparin (Holt and Bramanti, 1991). Moreover, by a multitude of complex and interconnected mechanisms, *P. gingivalis* can contribute to alveolar bone loss via stimulating bone resorption, inducing bone destruction and inhibiting bone formation (Holt and Bramanti, 1991). *P. gingivalis* lipopolysaccharide (LPS) can activate osteoclasts directly and cause the release of prostaglandin E_2 and of the cytokines IL-1β and TNF-α from macrophages, monocytes and fibroblasts (Lindemann et al., 1988, Bramanti et al., 1989, Sismey-Durrant and Hopps, 1991, Yamanji et al., 1995, Wilson et al., 1996c, Roberts et al., 1997). These compounds are known to be potent mediators of bone resorption and can inhibit collagen synthesis by osteoblasts and induce the production of host metalloproteases that destroy connective tissue and bone (Holt and Bramanti, 1991, Havemose-Poulsen et al., 1997). Of all the major immune mechanisms which may be encountered within a periodontal pocket, polymorphonuclear leukocytes (PMNs) seem to play a major role in the prevention of overgrowth of the sub-gingival microbiota (Darveau et al., 1997). *P. gingivalis* impinges upon almost all aspects of PMN recruitment and activity. Neutrophil chemothaxis is inhibited by low molecular weight fatty acids, such as succinic acid, which are produced by the organism (Rotstein et al., 1985). Succinate may also act by reducing the intracellular pH of neutrophils (Rotstein et al., 1985). *P. gingivalis* can also immobilise PMN responses to chemotactic peptides by the depolarisation of PMN membranes (Novak and Cohen, 1991).

1.2.7 Risk factors associated with periodontal disease

Periodontal disease may be exacerbated by several known factors, these may be intrinsic or extrinsic to the affected patient. Diabetes has been identified as a condition, which increases the likelihood of an individual developing periodontal disease. Diabetics may respond differently to pathogenic organisms due to vascular changes, PMN dysfunction and impaired immune regulation. (Salvi et al.,1997, Scannepieco et al.,1998). Smoking has also been identified as a risk factor in periodontal diseases (Salvi et al.,1997). Tobacco usage has been attributed to poor response to therapy and the increased incidence of recurrence (Martinez-Canut et al.,1996). Smoking also affects the immune response impairing chemotaxis and phagocytosis by neutrophils, decreased antibody production and elevated levels of TNF-α (Salvi et al., 1997, Bostrom et al.,1998). Individuals may also be genetically more disposed to a periodontal disease, this may be related in some instances to immune dysfunction due to a heritable disorder (Greenstein et al., 2000). Patients that are HIV seropositive or have progressed to AIDS are also at elevated risk of developing a periodontal disease which in some instances may be particularly aggressive (Greenstein et al., 2000).

1.3 Biofilm Control

1.3.1 Mechanical debridement of dental plaque

The primary way in which periodontal diseases are treated clinically is the use of mechanical force to remove both supra-gingival and sub-gingival plaque as well as calculus from the disease lesion. This mechanical debridement of the infected tissue is known as planing and root scaling. This treatment

methodology dates to the ancient Egyptians, and is still the preferred treatment of periodontal disease by clinicians in the 21st century. Scaling may be defined as the removal of both dental plaque and calculus supra- and sub-gingivally to the junctional epithelium. Root planing involves the thorough removal of the remaining plaque and bacterial products including LPS and toxins from the pocket cementum interface such that the resultant surface is smooth and devoid of any bacteria (Genco et al., 1990, Hoag et al., 1990, Jenkins et al., 1999). This treatment is extremely effective in treating periodontal diseases, hence its longevity (Mombelli et al., 2004). Mechanical removal of plaque and calculus coupled with scrupulous oral hygiene is sufficient in many instances in halting disease progression and concomitant attachment loss (Mombelli et al., 2004).

1.3.2 The use of antimicrobials in periodontal treatment

Antimicrobial compounds are currently widely used in the control of dental plaque and are hence useful in the prevention and/or treatment of periodontal diseases. Antimicrobial compounds are used as adjuncts to scaling and root planing. There are many chemotherapeutic compounds utilised by clinicians, one of the most ubiquitous being chlorhexidine. Chlorhexidine is a *bis*-biguanide and is a cationic compound, this results in it being rapidly attracted to the negatively-charged bacterial cell surface. After adsorption, the integrity of the bacterial cell membrane is altered, this results in a reversible leakage of bacterial low molecular weight components at low dosage or more severe membrane damage at higher concentrations (kuyyakanond et al., 1992). Chlorhexidine is a potent anti-plaque compound, and due to its cationic

nature, readily binds to enamel or to the nascent salivary pellicle, greatly increasing its residence time and hence bactericidal activity (Pratten et al., 1998a). However, administered chlorhexidine is of limited use in the treatment of periodontal disease. It poorly penetrates periodontal pockets and hence has a limited bactericidal effect on sub-gingival organisms. (Ciantar., 1995). The efficacy of chlorhexidine against biofilm-bound bacteria has been investigated by Pratten et al. The results of these studies show that whilst chlorhexidine may achieve a 90% reduction in the viable count of bacteria in the biofilm upon initial application, viable counts quickly recover to pre-pulsing levels (Pratten et al., 1998a).

Povidone-iodine is an antimicrobial which is microbicidal for Gram-positive and Gram-negative bacteria, fungi, mycobacteria, viruses and protozoa (Schreier et al., 1997). The antibacterial activity of povidone-iodine is due to the oxidation of amino, thiol and phenolic hydroxy groups in amino acids and nucleotides and its interaction with unsaturated fatty acids in cell walls and membranes (Quirynen et al., 2002). Significant, but small, additional effects have been reported with the use of povidone-iodine in conjunction with sub-gingival debridement. Immediately after pocket irrigation with a diluted povidone-iodine solution (0.2% free iodine), an appreciable antibacterial effect against black-pigmented, Gram-negative anaerobic rods can be detected. The proportions of spirochaetes and motile organisms are even reduced until 26 weeks after application (Nakagawa et al., 1990). Hence, it is possible to conclude that povidone-iodine seems to be potentially a promising antimicrobial product in periodontics (Quiryen et al., 2002).

1.3.3 Antibiotics

Antibiotics have been utilised extensively in the treatment of periodontal diseases. They are of particular use to the clinician when treating periodontal infections that prove to be refractory to traditional mechanical debridement coupled with scrupulous oral hygiene. Antibiotics may be either systemically administered or applied locally. The method of application depends on the nature of the periodontal lesion and the level of patient compliance. Several antibiotic compounds are routinely administered systemically including tetracycline and its semi-synthetic derivatives minocycline and doxycycline (Jenkins et al., 1999). Systemically supplied minocycline is commonly utilised against periodontal pathogens due to its relatively long serum half-life, low rate of urinary excretion and concentration in GCF 2 to 5 times higher than that found in serum (Ciancio et al.,1980). Minocycline has been shown to inhibit a number of periodontopathogenic species at concentrations clinically achievable, notably some black-pigmented anaerobes had somewhat higher minimum inhibitory concentrations (O'Connor et al., 1990).

Metronidazole is a commonly administered antibiotic, topical application is preferable to systemic administration as it avoids complications such as superinfection and gastrointestinal intolerance, it is also possible to deliver a higher concentration than that used systemically (Jenkins et al., 1999). Metronidazole was introduced for the treatment of periodontal infections due to it being retained by obligately anaerobic bacteria, this then leads to cell death (Muller at al., 1977) by interfering with the synthesis of nucleic acids (Walker, 1992). The metronidazole gel prepared for sub-gingival application

consists of a bioadsorbable delivery device containing 25 % metronidazole benzoate in a matrix formed from a mixture of glycerol monooleate and sesame oil (Norling et al., 1992). The decay of metronidazole over time in the crevicular fluid follows an exponential curve with a reservoir maintained for up to 12 hours (Stoltze et al., 1992). After 24 hours, the metronidazole concentration in the crevicular fluid still remains above the minimum inhibitory concentration for 50 % killing of key periodontal pathogens (Stoltze, 1995). The effect of Metronidazole gel applied as an adjunct to scaling and root planning has been scrutinized, however no additional benefits were apparent when scaling and root planning was combined with the application of metronidazole over and above what was clinically achieved via mechanical debridement alone; this despite repeated (5 times in 1 week) gel application (Riep et al., 1999).

Tetracycline-HCl is a bacteriostatic compound that inhibits bacterial protein synthesis and, as such, requires significantly longer exposure time than, for example, metronidazole or chlorhexidine (Tonetti, 1998). However, it possesses the ability to bind to the hard tissue walls of periodontal pockets thus establishing a drug reservoir (Christersson et al., 1993). Tetracycline releasing devices have been used mainly in trials of periodontitis patients. Of these, the most widely used is the Actisite periodontal fibre (ALZA, Palo Alto, CA, USA; Solco, birsfelden, Switzerland), a mono-lithic thread of biologically inert, non-resorbable plastic copolymer containing 25 % tetracycline hydrochloride powder (Mombelli et al., 2004). The fibre is then packed into the periodontal pocket , secured with a thin layer of cryanoacrylate adhesive

and left *in situ* for 7 to 12 days (Tonetti et al., 1990). When used in conjunction with planing and root scaling, locally administered tetracycline resulted in an appreciable probing depth reduction and/or attachment gain, in addition maintenance patients who harboured non-responding sites seemed to benefit from the locally applied tetracycline (Kinane et al., 1999). It appears that the application of tetracycline fibres in combination with rigorous and repeated sub-gingival debridement reduces the clinical signs of periodontitis and leads to an increase in attachment levels (Vandekerckhove et al., 1997). The clinical improvements evident when tetracycline fibres are employed in conjunction with mechanical debridement may stem from its substantivity and improved accessibility of the sun-gingival milieu due to fibre removal prior to planing and root scaling (Quirynen et al., 2002).

The local delivery of antibiotics proffers several advantages over their systemic use. It is known that the systemic use of antibiotics may result in a whole range of adverse effects such as hypersensitivity, nausea, diarrhoea, gastrointestinal intolerance, candidiasis, rashes and an unpleasant taste (Walker, 1996). However, this is not to imply that local administration should not be approached with the same caution that systemic antibiotic use is afforded. It is known that a patient with an allergy to a particular drug will still show a reaction even after localised application; indeed the locally applied compound may be absorbed such that this may result in an appreciable concentration of the compound in the patients' serum (Rapley et al., 1992). Moreover there is not a wealth of data available on the effects, if any, of sub-gingival slow release devices on the microbiota of the gastrointestinal tract

(Quirynen et al., 2002). Indeed, the dearth of published data has led to suggestions of the possible spread of antibiotic resistance genes and the spectre of multi-drug resistance after local antibiotic application (Kilian et al., 1995).

1.4 Methods for the *In vitro* modelling of biofilms

The advances that have been made in understanding the complex three-dimensional structures and elucidating the multitude of interactions which occur within the confines of a bacterial biofilm have been achieved using a number of methodologies. It is always preferable to study the biofilm either *in vivo* or *in situ*, the most pertinent data may always be garnered from studying the biofilm within the confines of its natural environment. However, cultivating biofilms *in vitro* offers benefits to the investigator, variation is inherent within the natural environment, *in vitro* cultivation allows the precise manipulation of the conditions in which the biofilm is cultivated and maintained, hence limiting variation. Conditions such as temperature, atmospheric environment, nutrient source and availability, substratum and inocula may be altered accordingly, allowing the precise identification of cause and effect relationships, which may be monitored via the biofilm itself. The different methodologies used to cultivate biofilms *in vitro* have been categorised (Brown et al., 1993) and a hierarchy is apparent from the very simple through to complex models which allow precise control of a range of growth parameters. There are the closed growth models, continuous growth models which are not steady state through to the steady state continuous growth models (in terms of biomass). The closed growth systems are rather limited in that they possess a finite nutrient

supply and no provision is made for removal of waste products generated by the cells. An example of such a system is the growth of biofilms directly on a solid medium such as an agar plate, or growth on a membrane filter which is in contact with the solid medium. The closed system may also refer to a solid/liquid interface, here the substratum used to generate the biofilm is immersed in a liquid nutrient. The non-steady state continuous growth models allow for the unrestricted cultivation of biofilms, continuous culture systems are often used to generate planktonic cells, these then form a sessile community upon the substratum surface. However, these biofilms are not in a steady state, biomass will continue to accumulate, altering the available nutrient supply. An example of such a system is the Robbins' device, this system was adapted and called the modified Robbins' device (McCoy et al., 1981). The system is constructed from an acrylic block and allows for multiple sampling, sampling ports contain sampling plugs which may be removed and replaced aseptically, culture effluent may be pumped through the block housing unit, typically from a continuous steady state culture, passing over the sampling plugs on which biofilm formation occurs. The modified Robbins' device has been used to model a range of systems including soft tissue infections (Deretic et al., 1989). A problem associated with such systems is the variability of growth rate over time which is due to fluctuations in the available nutrient supply. When investigating the effect of antimicrobials upon the biofilms it becomes difficult to distinguish between the effects of the antimicrobial and bacterial growth rate effects resulting from a depleting nutrient supply.

1.4.1 The Constant Depth Film Fermentor (CDFF)

The constant depth film fermentor is an example of a continuous growth model that allows growth rate associated problems to be overcome, this apparatus was first described by Peters and Wimpenny in 1988. A good *in vitro* model should incorporate features which allow it to be as flexible as possible and allowing maximal control over both biotic and abiotic conditions within the model. To this end the CDFF appears to be ideally suitable being sterilizable and providing for the aseptic removal of samples. It is suitable for investigations of natural and defined microbial consortia and indeed single species (Dibdin et al., 1999). The CDFF consists of a cylindrical glass vessel, this is sealed top and bottom via stainless steel end plates. Within the CDFF there is a stainless steel turntable, this turntable is capable of holding 15 polytetrafluoroethylene (PTFE) pans. These pans contain 5 PTFE plugs which are 5 mm in diameter. The plugs may be recessed to a chosen depth to reflect the *in vivo* situation or substrata may be placed on top of the plugs to more accurately mimic the surface interface *in vivo,* typically when modelling the non-shedding surfaces of the oral cavity hydroxylapatite is used. Two PTFE scraper blades are situated immediately above the turntable, their dual role being the smearing of nutrients over the biofilm surface and the exertion of shear forces upon the biofilm/air (or biofilm/anaerobic gas depending on operating conditions) interface thereby maintaining a constant depth. The upper end plate contains ports allowing the entry of sterile medium, gas, inocula and aseptic sampling of the fermentor, whilst the lower end plate harbours a port to allow drainage of the effluent. The CDFF itself is small enough to allow it to be housed within an incubator if appropriate. The

advantages of using this fermentor system are multiple, including the generation of large numbers of samples permitting statistical analysis and the controlled pulsing of both media or antibacterial compounds, multiple substrata may be investigated and substrata may be pre-treated before experimental commencement to allow the effect upon biofilm formation to be determined (Wilson., 1996b).

The CDFF has been previously employed to model the supra-gingival milieu within the oral cavity. Multi-species biofilms modelling inter-proximal plaque were grown on a hydroxylapatite substratum for the purposes of analysing the effects of chlorhexidine on oral biofilm viability and structure (Hope and Wilson, 2004). The CDFF has also been employed to culture multi-species oral biofilms on an enamel substratum from an inoculum composed principally of pooled human saliva (Pratten et al.,2000a, Pratten et al., 2003). Pratten et al., 2003 employed both culture and cloning techniques to quantify the microbiota cultivated within the model. The polyphasic approach resulted in the identification of 36 different bacterial species from both the inoculum and biofilms. All of the isolates and clones detected with the exception of *Bacillus subtilis* were considered to be part of the normal oral flora. Pratten et al., (2000a) subjected the multi-species biofilm to a differential feeding regime whereby the structures of sucrose-supplemented and non-supplemented biofilms were scrutinised using confocal microscopy. The biofilms contained structural motifs such as voids and channels consistent with what has been observed in studies of *in vivo* dental plaque (Pratten et al., 2000a) both

studies demonstrate that the CDFF is capable of generating biofilms which may be considered comparable to those found *in vivo* within the oral cavity.

1.5 Photodynamic therapy

The application of light for therapeutic purposes is rooted in antiquity. It was used in ancient Egypt, India and China to treat a wide range of dermatological complaints; it was even employed in the treatment of psychosis (Spikes, 1985, Epstein, 1990). The ancient Greeks advocated exposing one's whole body to the sun, which was known as heliotherapy in the treatment of differing diseases and this developed into a popular past-time (Ackroyd et al., 2001). A Greek physician by the name of Herodotus extolled the virtues of sun exposure for therapeutic purposes and is regarded as the father of heliotherapy (Ackroyd et al., 2001). In the eighteenth and nineteenth centuries in France, sunlight was employed in the treatment of a plethora of complaints which included tuberculosis, rickets, scurvy, rheumatism, paralysis, edema and muscle weakness (Cauvin, 1815). Phototherapy was further developed by Niels Finsen, at the turn of the last century he successfully employed red light for the treatment of smallpox, the application of the red light prevented suppuration of the pustules (Finsen, 1901). Phototherapy involves the utilisation of light in the treatment of disease, photochemotherapy involves the application of a photosensitising agent followed by illumination with an appropriate light source (Ackroyd et al., 2001). Again, this form of therapy has been practised for several thousand years. Over 3000 years ago the Indians used psoralens in the treatment of vitiligo (Fitzpatrick et al., 1959). However, the concept that cell death may be

induced via the concerted action of light and chemicals is a relatively recent one, being recognised for only c. 100 years (Ackroyd et al., 2001). A medical student by the name of Oscar Raab, as part of his study on the malaria-causing protozoa, discovered that the combination of acridine red and light had a lethal effect on paramecia (Raab, 1900). The experiment had been conducted during a thunder storm when ambient light conditions had been abnormal leading to this chance discovery. It was later demonstrated that the effect exerted by light in combination with acridine was greater than that of either acridine alone or light alone. Raab concluded that it was not light per se but rather some product of the fluorescence that resulted in *in vitro* activity. Raab hypothesised that this effect was caused by the transfer of energy from light to the chemical, analogous to that seen in plants after the absorption of light by chlorophyll (Von Tappeiner, 1900). Von Tappeiner went on to demonstrate the requirement of oxygen for photosensitisation reactions to proceed (Von Tappeiner and Jodlbauer, 1903), and in 1907 introduced the term 'photodynamic action' to describe this phenomenon (Von Tappeiner and Jodlbauer, 1907).

1.5.1 Mechanism of action

Photodynamic therapy mediates bacterial killing, or in the case of oncology, tumour necrosis, by two main photochemical pathways. The two proposed pathways being designated type I and or type II reactions. Compounds used to achieve photosensitisation are typically aromatic and extremely efficient at producing long-lived triplet excited states. Characteristics which are important in a photosensitiser and are directly related to its electronic structure include

lipophilicity (helping to determine where the compound is likely to localise in the organism), maximum wavelength of absorption and intensity of absorption (Wainwright., 1998). In addition, a photosensitiser should ideally only exert its bactericidal effect on illumination with the appropriate light source and should not demonstrate dark toxicity. Production of the excited triplet states is due to the elevation of electrons into a higher energy level (Wilson.,1994a) upon stimulation by polychromatic (such as visible light) or monochromatic light (produced by lasers) which is essential if photosensitisation is to occur (Wainwright., 1998). The proposed type I reaction is characterised by the interaction between the triplet state photosensitiser with oxidizable compounds resulting in the abstraction of hydrogen or electron transfer producing reactive radicals or radical ions. A type II reaction is one where energy transfer occurs between the excited triplet state photosensitiser and molecular oxygen, this results in the formation of highly unstable, and hence reactive, singlet oxygen (Burns et al., 1996, Martin et al., 1987, Phillips., 1997., Wilson.,1994a).

The type II mechanism is one that has been heavily implicated in the photochemical inactivation of microorganisms. The effect of the photosensitiser toludine blue O (TBO) a tricyclic heteroaromatic compound (maximal absorbance 633 nm, its structure is shown in figure 1.6), stimulated using a HeNe laser light emitting monochromatic collimated light at 632.8 nm was determined upon the organism *P. gingivalis*. The results showed that in the presence of deuterium oxide, known to prolong the half-life of singlet oxygen, a considerable increase in sensitiser efficacy was found, indirectly

implicating a type II mechanism (Bhatti et al., 1998). It was also found that the sensitiser was concentrated in the outer membrane of *P. gingivalis* suggesting that TBO may act preferentially on this structure (Bhatti et al., 1998). A similar study using TBO and a HeNe laser on the cariogenic bacterium *Streptococcus mutans* demonstrated increased photosensitisation in the presence of deuterium oxide and no detectable photosensitisation under anaerobic conditions. Addition of oxygen scavengers to the system greatly decreased photosensitisation, all these observations strongly implicate type II reactions in the photosensitisation of microorganisms (Burns et al., 1997). Studies on the photosensitisation of *Escherichia coli* using TBO led the investigators to conclude that TBO is membrane-active (Wakayama et al., 1980). Experiments using yeast cells have also implicated plasma membrane damage as the primary mechanism of lethal photosensitisation (Ito.,1977a, Ito et al.,1977b). These results demonstrate that TBO is membrane-active, this being the cellular target disrupted via photosensitisation utilising TBO.

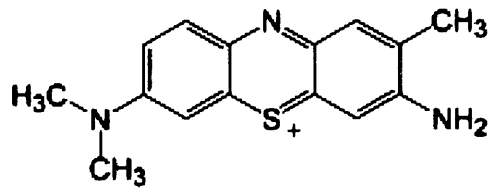


Figure 1.6: A diagram of the structure of TBO.

1.5.2 Applications of PDT

Photodynamic therapy is currently utilised in the field of oncology for the treatment of a range of cancers. Photodynamic therapy has been used clinically for the treatment of bladder cancer, lung cancer and various malignant diseases of the skin and the upper respiratory tract (Schuitmaker et al., 1996). The photosensitising drug is administered intravenously and is selectively taken up and retained by malignant cells (Carruth., 1998). The photosensitiser most frequently used is haematoporphyrin derivative (HPD) or more recently its active component dihaematoporphyrin ether (DHE) in combination with red light at 630 nm (Carruth., 1998). Photodynamic therapy has been demonstrated to be effective in the sterilisation of human derived blood and blood products. It has been shown that the tricyclic heteroaromatic compound methylene blue at a concentration of 1 μM sensitises, among others, hepatitis B and C as well as human immunodeficiency virus-1 (Mohr et al., 1997). Photodynamic therapy utilising aluminium disulphonated phthalocyanine ($AlPcS_2$) was able to effectively photosensitise methicillin-resistant *Staphylococcus aureus* (MRSA), a frequent cause of noscomial infections (Griffiths et al., 1997). Whilst polychroamatic light allied to methylene blue was shown to be effective against a wide range of bacteria associated with human skin and opportunistic pathogens such as *Candida albicans* (Zeina et al., 2001). Photosensitisation of *Helicobacter pylori* with an assortment of photosensitisers including TBO has been demonstrated *in vitro* (Millson et al.,1996), this is significant as *H. pylori* has been implicated as the causative agent of gastric ulcers and adenocarcinomas and is currently treated via administration of systemic antibiotics or surgery.

1.5.3 Use of Photodynamic therapy in the oral cavity

Photodynamic therapy has been demonstrated as having a bactericidal effect against many of the organisms that are the causative agents of dental caries and periodontal diseases. Photodynamic therapy has been shown to be effective against a wide range of planktonic single species oral bacteria including *S. sanguinis* which was shown to be susceptible to a wide range of photosensitisers; TBO, haematoporphyrin ester, thionin and methylene blue in conjunction with HeNe laser light (Wilson et al., 1993a). Toluidine blue and HeNe laser light has been demonstrated as having a bactericidal effect on biofilms composed of *S. sanguinis* (Wilson et al.,1996a). It has been demonstrated that both TBO and $AlPcS_2$ in combination with either a HeNe or gallium aluminium arsenide (GaAs) laser respectively, effectively photosensitised bacteria composing supra-gingival plaque samples (Wilson et al., 1995c). This is significant due to the known recalcitrance of biofilms to the application of bactericidal or bacteriostatic compounds. Photodynamic therapy has been shown to effectively kill a range of bacteria associated with caries and dental plaque formation as well as having appreciable activity against biofilm-bound bacteria.

1.6 Aims and Objectives

This introduction has given a brief summary of the problems associated with treating periodontitis. The bacteria indigenous to the oral cavity possess a prodigious ability to form biofilms, these biofilms differ in composition depending upon whether they form supra- or sub-gingivally. However, what is apparent is the recalcitrance of the bacteria to many anti-microbial

compounds when they are present as biofilms; this recalcitrance certainly helps to explain the refractory nature of certain forms of periodontal diseases. Many of the current treatments which are available today may be successful in reducing the bacterial load within a periodontal pocket, but are unable to reduce numbers of the putative pathogenic bacteria such that the disease is effectively eliminated. Indeed, there are several disadvantages associated with the use of anti-microbial compounds commonly administered, including problems of resistance. Therefore, PDT may represent a new and improved method of treating periodontitis, certainly as an adjunctive in combination with more traditional mechanical debridement. Although the use of PDT for the treatment of bacterial infections is still in its infancy, it has been successfully used in the treatment of certain carcinomas and certainly shows huge potential in the field of infectious diseases.

The aims and objectives of this study can be summarised as follows:

1. To identify a suitable photosensitiser for use in conjunction with a HeNe laser light source.
2. To develop a laboratory model capable of producing large numbers of biofilms that mimicked, as regards composition and structure, the sub-gingival plaques found *in vivo*.
3. To determine whether the chosen photosensitiser in conjunction with HeNe laser light can effectively photosensitise periodontopathogenic bacteria when cultivated as biofilms.

4. To conduct a comparative analysis of the efficacy of lethal photosensitisation when compared to a commonly prescribed anti-microbial such as chlorhexidine digluconate.

CHAPTER TWO

MATERIALS AND METHODS

2. Materials and methods

2.1 Organisms

2.1.1 Growth of single species

2.1.1.1 *Streptococcus sanguinis*

The strain used was *Streptococcus sanguinis* NCTC 10904 (unless stated otherwise) which was maintained by sub-culturing on tryptone soya agar (Oxoid Ltd., Basingstoke, UK) every week. For experimental purposes, the organism was grown overnight (16 h) in tryptone soya broth (Oxoid Ltd.) at 37°C in an anaerobic environment consisting of 80% N_2, 10% CO_2 and 10% H_2 (Don Whitley Scientific Ltd., Shipley, UK).

2.1.1.2 *Porphyromonas gingivalis*

The strain used was *Porphyromonas gingivalis* W50 which was maintained by sub-culturing on Fastidious Anaerobe Agar (FAA) (Bioconnections, Leeds, U.K) containing 5 % (v/v) defibrinated horse blood (E & O Laboratories, Bonnybridge, U.K). For experimental purposes the organism was grown in Bacteroides medium (BM) broth for 2 days at 37°C in an anaerobic environment. The composition of the BM broth was as follows: tryptone soya broth 10 g (Oxoid Ltd), protease peptone 10 g (Oxoid Ltd.), yeast extract 5 g (Oxoid Ltd.), glucose 5 g (Sigma Ltd., Poole, U.K), sodium chloride 5 g (Sigma Ltd.), and cysteine-hydrochloride 0.75 g (Sigma Ltd.) per litre of distilled water. The pH was adjusted to 7.5, and the broth was autoclaved at 121° C for 15 min. The medium was supplemented with haemin

(Sigma Ltd.) and menadione (Sigma Ltd.) prior to use so that the final concentrations were 5 μg/ml and 0.5 μg/ml, respectively.

2.1.1.3 *Fusobacterium nucleatum*

The strain used was *Fusobacterium nucleatum* F28 (a clinical isolate from the Eastman Dental Institute) which was maintained by sub-culturing on FAA supplemented with 5 % (v/v) defibrinated horse blood every week. For experimental purposes the organism was grown in brain-heart infusion (BHI) broth (Oxoid Ltd) for 24 hours at 37° C in an anaerobic environment.

2.1.1.4 *Actinobacillus actinomycetemcomitans*

The strain used was *Actinobacillus actinomycetemcomitans* HK 1651 which was maintained by sub-culturing on FAA supplemented with 5 % (v/v) defibrinated horse blood every week. For experimental purposes the organism was grown on BHI broth for 2 days at 37° C in an anaerobic environment.

2.1.1.5 *Actinomyces viscosus*

The organism used was *Actinomyces viscosus* (a clinical isolate) which was maintained by sub-culturing on FAA supplemented with 5 % (v/v) defibrinated horse blood every week. For experimental purposes, the organism was grown in BHI broth for 24 h at 37° C in an anaerobic environment.

2.1.2 Collection of subgingival plaque samples and pooled saliva samples

2.1.2.1 Subgingival plaque samples from patients with periodontitis

Subgingival plaque samples were obtained from patients attending the Eastman Dental Hospital for treatment for moderate-to-advanced adult periodontitis. Subgingival plaque samples were collected from these patients, each sample contained the contents of at least two periodontal pockets. Each sample was suspended in 1 ml of Reduced Transport Fluid (RTF) as a cryoprotectant and these were then stored in a freezer (Lab Impex Research, Middlesex, U.K) at –70º C. The composition of RTF was as follows: Stock solution 1: dibasic potassium phosphate 0.6 g (K_2HPO_4) (Sigma Ltd), distilled water 100 ml. Stock solution 2; potassium chloride 1.2 g (Sigma Ltd.), ammonium sulphate 1.2 g (Sigma Ltd.), monobasic potassium phosphate (KH_2PO_4) 0.6 g (Sigma Ltd.), 99ml distilled water, $MgSO_4$ stock solution 1 ml ($MgSO_4$ 2.5 g (Sigma Ltd.), distilled water 100ml). Sodium carbonate solution; sodium carbonate 0.8 g (Sigma Ltd.), distilled water 10 ml, to prepare 100 ml of RTF, 7.5ml stock solution 1, 7.5ml stock solution 2, 0.5 ml sodium carbonate solution and 80 ml of distilled water. This was then autoclaved at 121° C for 15 min, when cooled to room temperature a filter sterilised solution of dithiothreitol (DTT, Sigma Ltd.) (0.02 g of DTT in 5 ml of distilled water) was added (Syed et al., 1972). The samples were thawed and centrifuged at 8,000 g in a desktop centrifuge (Eppendorf 5810R, Eppendorf, Cambridge, U.K) for 10 min, the RTF (upper layer) was then removed. The samples were suspended in 20 ml of pre-reduced BHI broth with

10% glycerol. These were homogenised and sub-divided to give 20 x 1 ml aliquots and stored at -70° C.

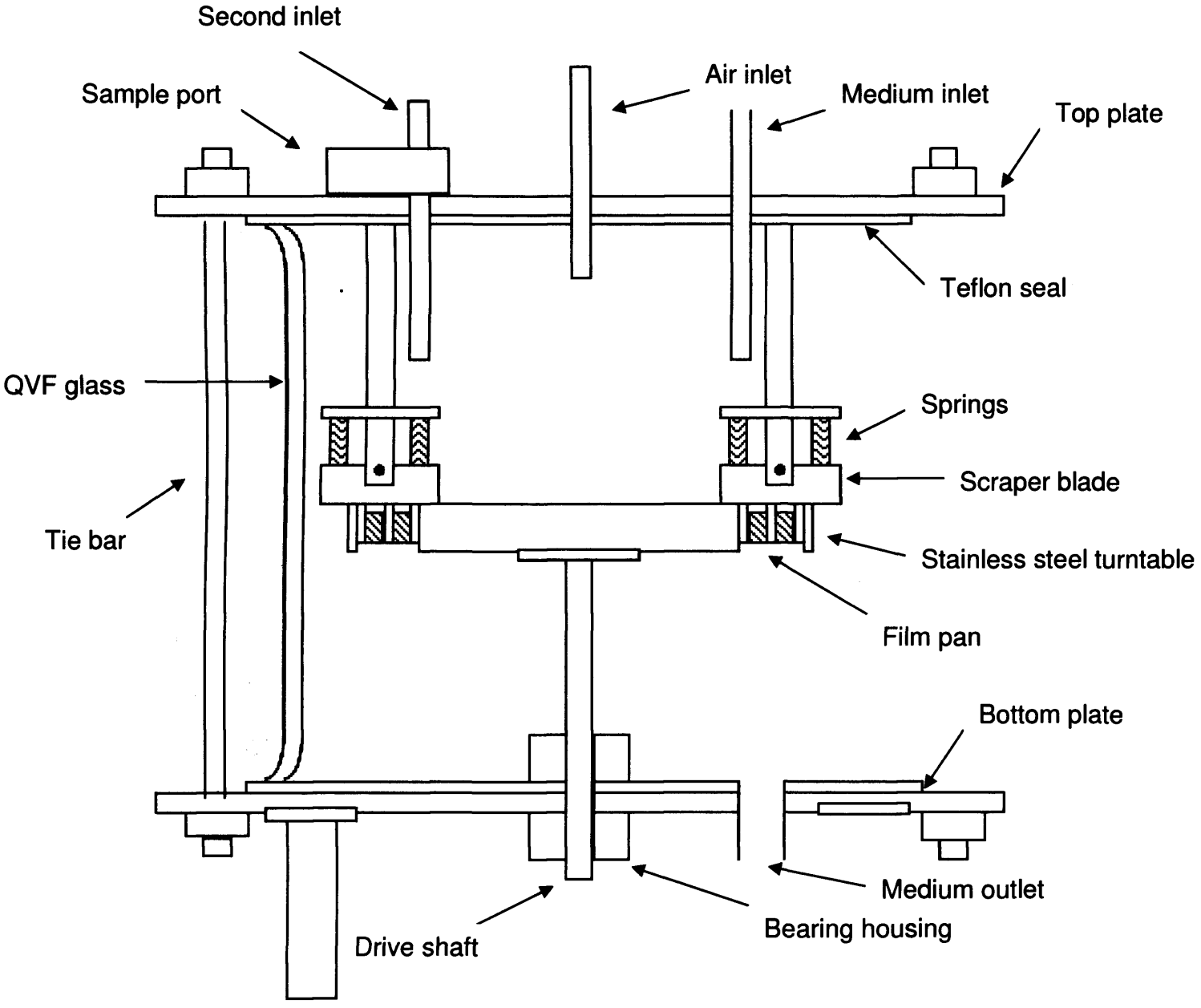
2.1.2.2 Preparation of pooled saliva samples

Salivary samples were taken from ten members of the microbiology department, Eastman Dental Institute. Three millilitres was taken from each individual resulting in 30 ml of pooled saliva. To this was added 30 ml of BHI broth with 10% glycerol (Sigma Ltd.), the glycerol being a cryoprotectant. This was vortex mixed vigorously for 1 minute to homogenise the sample. Aliquots of 1 ml were then dispensed into 50 sterile cryotubes. These were then stored at – 70° C.

2.2 The constant depth film fermentor (CDFF)

The CDFF (John Parry Jones Engineering, Cardiff, U.K) is an autoclavable device (Peters et al., 1988), and enables the production and aseptic removal of 75 biofilms of defined thickness. The CDFF possesses 15 polytetrafluoroethylene (PTFE) pans that are inserted into the stainless steel turntable. Each pan has 5 plugs, the plugs themselves may be recessed to allow biofilm formation or alternatively a substratum may be inserted over the plugs and recessed to a known depth. Hence the design of the CDFF enables the generation of large numbers of reproducible biofilms. Two scraper blades remove excess medium, and upon biofilm formation maintain a constant biofilm thickness. The scraping produced by the blades helps to mimic the shear forces found within the oral cavity.

Figure 2.1: A cross-section of the CDFF.



2.2.1 Preparation of hydroxylapatite discs

The substratum for subgingival plaque is enamel/cementum, the mineral component of which is similar to hydroxyapatite. Hydroxylapatite discs were, therefore, used as the substratum for biofilm growth in the CDFF. Hydroxylapatite powder (Plasma Biotal Ltd, Derbyshire, U.K) was added to fill a mould (Perkin-Elmer, U.S.A) of 5 mm diameter. The powder was subjected to increasing mechanical pressure up to 0.25 of a tonne which was then released immediately. This resulted in the formation of a disc with a 5 mm diameter for insertion into the CDFF. This process was repeated to produce 75 such discs. The newly-pressed discs were placed in a furnace (Lenton Thermal Designs Ltd, U.K) at 1000°C for 1 h.

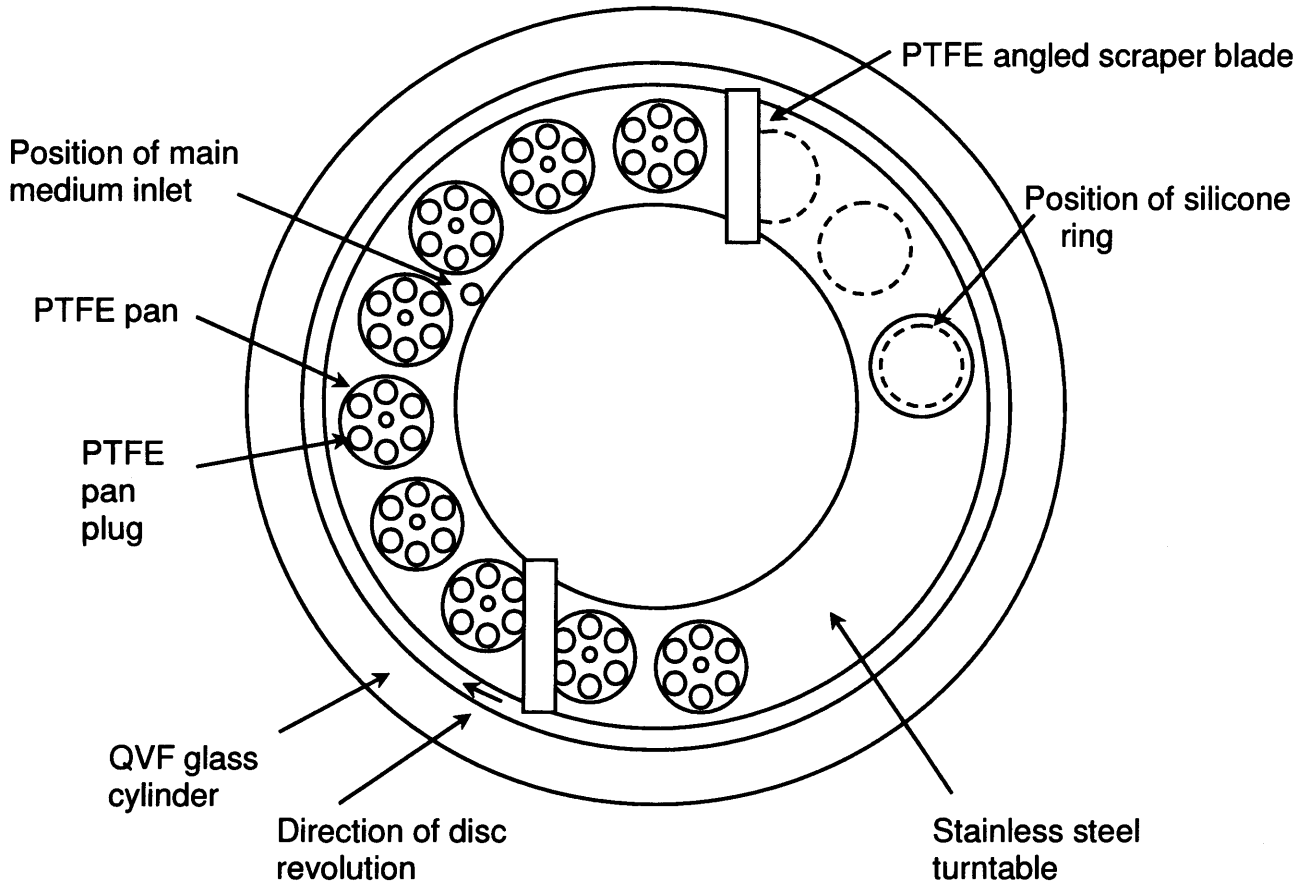


Figure 2.2: An overview of the CDFF looking down upon the steel turntable showing the arrangement of the PTFE pans

2.2.2. Assembly of the CDFF

The hydroxylapatite discs were inserted into the fermentor pans and recessed to 100 µm using a purpose-built tool. The fermentor was sealed using a silicon grease which was applied to both of the PTFE seals, this prevented leakage of the medium from the CDFF and also helped to maintain the atmospheric

conditions within the fermentor. Tubing was then connected to the CDFF to enable delivery of nutrients, microaerophilic gases and allow the removal of effluent. The CDFF was subsequently autoclaved at 121 °C for 30 minutes.

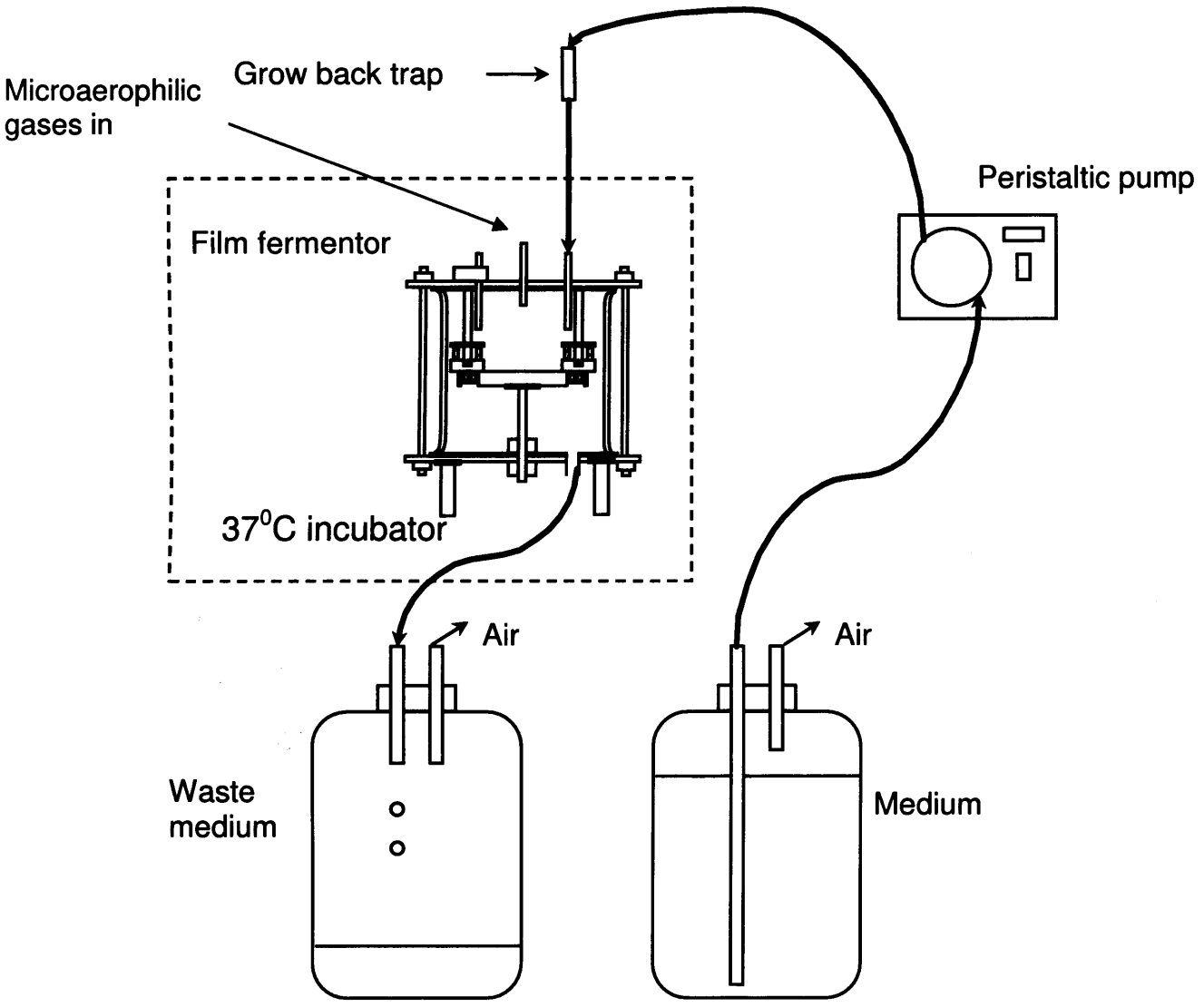


Figure 2.3: A schematic diagram showing the complete fermentor assembly

2.2.3 Growth medium

The medium used consisting of RPMI (60 % with L-glutamine, without phenol red) (Sigma Ltd.) and horse serum (40 %, this was not heat inactivated) (Oxoid Ltd.). This medium composition was chosen as an approximation of gingival crevicular fluid - the fluid component of the subgingival region in which subgingival plaque is bathed *in vivo*.

2.2.4 CDFF Inoculation

For each experiment, two 1 ml aliquots of the subgingival plaque samples were inoculated into 500 ml of 40% horse serum in RPMI medium. To inoculate the CDFF, this bacterial suspension was pumped (using a peristaltic pump [Watson and Marlow, Falmouth, U.K]) for 8 h at a rate of 1 ml/min into the CDFF

2.2.5 Gaseous conditions within the CDFF

The CDFF operated under microaerophilic conditions by continuous flushing (pressure 1 bar, flow rate through the CDFF 60 cm^3/min) with an atmosphere consisting of 95 % N_2, 3 % CO_2, 2 % O_2, the gas cylinder (BOC, Guildford, U.K) being connected to the CDFF via silicon tubing, this passed through a 0.22-µm filter (Whatman, Maidstone, U.K) before entering the fermentor.

2.2.6 Growth of multi-species biofilms using the CDFF

The medium used to enable growth of the microcosm plaques consisted of RPMI (60 %) / horse serum (40 %) supplemented with 0.5 µg/ml menadione and 5.0

μg/ml haemin. This was delivered to the inoculated CDFF at a flow rate of 0.1 ml/min, this flow rate being the lowest that may be used whilst preventing the desiccation of the biofilm. The CDFF was housed within an incubator at a constant 37°C, the CDFF was maintained under the constant conditions stated above.

2.2.7 Sampling of the CDFF

The pans were removed from the CDFF at the appropriate time interval via the use of a specially designed sampling tool. Before sampling this was wrapped in aluminium foil and autoclaved at 121 °C for 15 minutes. To sample, the turntable was stopped so as the pan wanted for sampling was directly under the sampling port. The sampling port was then flamed using 96% ethanol, and the sampling lid removed. The sample tool was then placed into the fermentor and screwed into the pan. The pan was quickly removed and the lid replaced. The pan was placed into a sterile universal container for transport. To remove the disc from the pan the PTFE plugs were forced upwards using sterile forceps, once the discs were revealed they were removed from the pan.

2.3 Bacteriological characterisation of multi-species biofilms

A disc (plus its associated biofilm) was placed in 1 ml of BM broth. The disc was vortex-mixed for 60 seconds to disrupt the biofilm. Ten-fold serial dilutions were then performed and duplicate 25 μl aliquots were spread over the surface of the following media:

FAA and Coloumbia Blood Agar (CBA) (Oxoid Ltd) both of which contained 5 % (v/v) defibrinated horse blood.

Selective media were also used to culture the following genera;

Actinomyces spp. were isolated on Cadmium fluoride-acriflavin-tellurite agar plates (CFAT) (Zylber et al., 1982), *Veillonella* spp. on Veillonella agar (VEI) (Difco, Detroit, MI, U.S.A), streptococci on Mitis Salivarius agar (MS) (Difco). To isolate Gram negative anaerobic species, FAA plus 2.5 µg/ml vancomycin (Sigma Ltd), 10 µg/ml nalidixic acid (Sigma Ltd) and 5 % defibrinated horse blood (v/v) (FAX) was used.

After incubation in an anaerobic cabinet for up to 7 days, with the exception of the Columbia agar which was incubated aerobically overnight, the colonies on each plate were counted and subjected to a number of confirmatory identification tests.

The confirmatory tests used were as follows:

a) Representative colonies were Gram-stained

b) Those suspected of being streptococci were subjected to catalase testing

c) Those suspected of being obligate anaerobes (i.e. *Veillonella* spp., Gram-negative anaerobes and black-pigmented anaerobes) were sub-cultured and tested for their ability to grow in a CO_2-enriched (5% CO_2) aerobic atmosphere

2.4 Growth of single species biofilms on membrane filters

2.4.1 Preparation of membrane filters

Five millimetre diameter discs were prepared from 0.45 µm cellulose nitrate filters (Sterilin Ltd., Hounslow, UK). These were autoclaved in phosphate buffered saline (Oxoid Ltd.) to prevent desiccation.

2.4.2 Biofilm production

The bacterium to be grown as a biofilm was grown overnight in the appropriate broth within the anaerobic cabinet. Upon successful overnight cultivation of the bacterium, the culture was removed and centrifuged at 700 g for 10 minutes in a desktop centrifuge (Eppendorf). The supernatant was removed, the bacterial culture was diluted in saline (0.85% w/v) to give an optical density of approximately 1 at 560 nm (Ultrospec 2000, Pharmacia biotech). The 5 mm diameter cellulose nitrate discs were then added to the Petri plates containing FAA. Each disc was inoculated with 25 µl of bacterial suspension. The plates were then placed in an anaerobic cabinet.

2.5 Confocal laser scanning microscopy (CLSM) of biofilms

The biofilms were placed into a miniature Petri dish, biofilm uppermost. Ten millilitres of chilled phosphate buffered saline (PBS) containing 10 µl of component A and component B (*Bac*Light™ bacterial viability kit, molecular probes, Oregon, USA) was carefully added to the Petri dish and allowed to cover the biofilms. This was then allowed to stand in the dark for 5 minutes to allow for

stain development and biofilm penetration. Scans were taken of the biofilms using a Leica DMLFS fixed stage microscope with a Leica TCS SP confocal scan-head. The objective lens was a 63x HCX water immersion dipping objective lens. These scans were performed by the CLSM staff at the department of Anatomy, University College London.

2.5.1 Analysis of the CLSM data

Intensity profiles were generated from the raw data for both the viable (488nm) and nonviable (568nm) channels using Image J computer software (Image J 1.22d, The National Institutes of Health, USA, http://rsb.info.nih.gov/ij/). To enable effective comparison between the live and dead channels, the data were normalised against the maximum image intensity for each channel. To obtain the relative intensity value, the normalised nonviable value were subtracted from the normalised viable value.

2.6 Susceptibility of single species organisms and biofilms to lethal photosensitisation.

2.6.1 Preparation of photosensitisers and the laser light source

2.6.1.1 Preparation of photosensitisers

The Toluidine blue O (TBO) (C.I. 52040), was obtained from Sigma Ltd, Poole, UK (sTBO, 90% pure). The Pharmaceutical grade TBO (pTBO, 99% pure) was supplied by Ondine Biopharma (Vancouver, B.C, Canada). The photosensitisers, methylene blue (MB), new methylene blue (NMB), 1,9-dimethyl methylene blue

(DMMB), chlorin e6, zinc phthalocyaninetetrasulfonic acid, tetrasodium salt and protoporphyrin IX disodium salt were all obtained from Dr Mark Wainwright (University of Leeds, U.K). The photosensitisers were weighed out on a Sartorius balance (Sartorius Instruments Ltd, Surrey, UK) and made up to the requisite concentration in sterile saline (0.85% w/v), and then filter-sterilised using a Nalgene 0.2 μm filter.

2.6.1.2 The laser

The laser used was a Helium/Neon (He/Ne) gas laser with a measured power output of 35 mW (Spectra-Physics, Darmstadt-Kranichstein, Germany). This emits radiation in a collimated beam, diameter 3.5 mm, with a wavelength of 632.8 nm. When irradiating biofilms the laser was used in conjunction with a lens to enable irradiation of the entire biofilm. The use of a lens resulted in the laser beam increasing in diameter from 3.5 mm to 7.0 mm.

2.6.2 Susceptibility of planktonic single species to photosensitiser/HeNe laser light

The overnight bacterial culture of *S. sanguinis* (unless otherwise stated) was centrifuged at 700 g for 10 minutes. This was diluted in saline (0.85% w/v) to give an optical density of approximately 0.2 at 560 nm, 100 μl aliquots of this bacterial suspension were transferred to wells of a 96-well, round-bottomed micro-titre plate (Sterilin Ltd.). An equal volume of the filter-sterilised solution of the photosensitiser was added to some of the wells (4 for each photosensitiser). Four millimetre

magnetic stirrer bars were added to each well, the plate was placed on a magnetic stirrer and half of the wells (2 for each photosensitiser) were exposed to light from a He/Ne laser. Suspensions treated in this way were designated (L+S+). The remaining wells (2 for each photosensitiser) were not exposed to laser light and these served as controls to determine the effect of each photosensitiser on bacterial viability in the absence of laser light (L-S+). Control wells (2) containing the bacterial suspension plus sterile saline in place of the photosensitiser solution were also irradiated to determine the effect of laser irradiation alone on bacterial viability (L+S-). A further 2 wells, identical to those described above, were prepared and these were not exposed to laser light (L-S-).

The number of viable organisms surviving in each well was then determined by viable counting, 10-fold serial dilutions were performed in BM broth and duplicate 50 µl aliquots were spread across FAA plates (unless stated otherwise). The resultant plates were incubated in an anaerobic environment at 37°C. The bacterial colonies were then enumerated.

2.6.3 Susceptibility of membrane grown biofilms to TBO/HeNe laser light

An FAA plate containing discs with associated biofilms was removed from the anaerobic environment 24 hours after seeding of the cellulose nitrate membranes. To a filter disc was added 10 µl of TBO (L+S+). A 30 s pre-exposure time was allowed before exposing the L+S+ disc to laser light. The L+S+ was then placed in 1 ml of sterile BM broth and vortexed for 60 s. Ten-fold serial dilutions of the vortex-disrupted biofilm were prepared in sterile BM broth

and duplicate 50 µl aliquots were spread over the surfaces of FAA plates. The same procedure was then repeated for a second biofilm.

In order to determine the effect of laser light alone on bacterial viability 10 µl of saline was added to another two biofilms (L+S-) a 30-second pre-exposure time was allowed to elapse before the discs were exposed to laser light as described above. The other controls consisted of biofilms treated with TBO but not exposed to laser light (L-S+) and biofilms which were neither treated with TBO nor exposed to laser light (L-S-). All plates were incubated in an anaerobic cabinet at 37° C for up to 7 days. The resulting colonies were counted.

2.6.4 Susceptibility of CDFF grown multi-species biofilms to TBO/HeNe laser light

Pans were removed from the CDFF and the biofilms tested for their susceptibility to TBO/HeNe laser light. During the morning of each sampling day, one PTFE pan containing 5 hydroxylapatite discs was removed aseptically from the CDFF and placed in a sterile universal container for transport. The discs were removed from the PTFE pan and placed in a 12-well flat bottomed plate (Sterilin Ltd.). To one disc, 10 µl of TBO was added (L+S+) to the other disc an equal volume of sterile saline (0.85%) was added (L-S- [C1]). The control well served to determine whether exposure to an aerobic environment for the duration of the experiment had any effect on bacterial viability. The discs were then left for a 5 minute pre-exposure time to allow the applied solutions to penetrate into the

biofilm. The L+S+ disc was exposed to light from a He/Ne laser. Immediately following this exposure, the remaining discs were then treated as follows. To one disc, 10 µl of TBO was added (L-S+). To the two remaining discs, 10 µl of sterile saline (0.85% w/v) was added. One served as a control disc to determine whether exposure to an aerobic environment for the duration of the experiment had an effect on bacterial viability (L-S- [C2] the summation and subsequent mean value of C1 and C2 formed the mean L-S-) the other was utilised to determine the effect of laser irradiation alone on bacterial viability (L+S-). During this exposure period, the L+S+ and L-S- (C1) discs were prepared for viable counting. Ten-fold serial dilutions were prepared in BM broth. Duplicate 25 µl aliquots were then spread over the surface of FAA plates. By this time, the second series of discs were ready for processing and these were prepared for viable counting in the manner described. The above procedure was also carried out on a separate pan during the afternoon of each sampling day. After incubation of these inoculated plates in an anaerobic cabinet for up to 7 days, the colonies on each plate were subsequently counted.

CHAPTER THREE

IDENTIFICATION OF A SUITABLE PHOTOBACTERICIDAL AGENT

3.1 Introduction:

Lethal photosensitization of bacterial cells has been achieved using a wide spectrum of sensitising agents (Wainwright.,1998b). It has previously been demonstrated that the phenothiazine dyes, particularly TBO, are excellent photosensitising agents of putative cariogenic and periodontopathogenic organisms (Wilson et al.,1993a,b,c). Recently a number of novel phenothiazine compounds have been described in the literature as possessing photobactericidal activity (Wainwright et al., 1997,1998a). In addition to the phenothiazines, other agents have been successfully employed for the lethal photosensitisation of both Gram-positive and Gram-negative bacteria (Martinetto et al., 1986, Nitzan et al., 1989, Malik et al., 1990, Minnock et al., 1996, Griffiths et al., 1997, Nitzan et al., 1998), moreover compounds such as the porphyrins have been utilised extensively as therapeutic agents in the Photodynamic Therapy (PDT) of various cancers (Van Hillegersberg., 1994). Due to the broad range of photosensitisers which have previously been shown to photosensitise an array of different bacteria, several promising compounds were chosen for a preliminary investigation; to assay their efficacy against an oral bacterium in conjunction with HeNe laser light. The candidate photosensitisers were subjected to an initial screening experiment against the oral commensal *Streptococcus sanguinis*. Lethal photosensitization may proceed through two photochemical pathways, the type II pathway being heavily implicated in the photoinactivation of bacterial cells. It is hypothesised that the type II mechanism generates singlet oxygen as the primary mediator of cell death. Uric acid is a

compound that readily scavenges singlet oxygen; therefore uric acid was incorporated into an assay to quantify singlet oxygen production, a predictive indicator of potential photosensitising efficacy of a compound. The screening against *S. sanguinis* coupled with the singlet oxygen production assay were used to identify a potentially suitable photosensitiser; this was then subjected to a more comprehensive determination of photosensitising ability against a range of both Gram positive and negative organisms.

3.2 Materials and methods

3.2.1 Growth of *Streptococcus sanguinis*

The method used was the same as that described in chapter 2, section 2.1.1.1, with the following exceptions:

1. The organism used was *Streptococcus sanguinis* NCTC 7863

3.2.2 Preparation of photosensitising agents

This was described in chapter 2, section 2.6.1.1

3.2.3 Susceptibility of *Streptococcus sanguinis* to photosensitiser/HeNe laser light

The method used was the same as that described in chapter 2, section 2.6.2, with the following exception;

1. Duplicate 50 µl aliquots of bacterial suspension were spread over the surface of tryptone soya agar plates (Oxoid Ltd).

3.2.4 Singlet oxygen production of the photosensitising compounds

The following protocol, used to quantify singlet oxygen yield was adapted from Fisher et al., (1998), any decrease noted in the absorbance of the uric acid solution at 293 nm upon exposure to laser light was considered to be directly proportional to the singlet oxygen yield of the photosensitiser. Uric acid (Sigma Ltd) was made up in phosphate buffered saline (PBS) (Oxoid Ltd) as a fresh stock solution to a concentration of 100 μg/ml on each day the assay was to be carried out. To 1.0 ml of phosphate buffered saline was added 500 μl of photosensitiser (concentration 81.7μm) and 500 μl of 100 μg/ml uric acid, this was then exposed to laser light for 60 seconds (L+U+S+). A series of control solutions were also prepared. To 1.5 ml of PBS, 500 μl of 100 μg/ml uric acid was added and was exposed to laser light for 60 seconds (L+U+S-), to 1.5 ml of PBS, 500 μl of photosensitiser was added and was exposed to laser light (L+U-S+) The absorbance of the assay plus control solutions was measured at 293 nm in a spectrophotometer both pre- and post – exposure to laser light and recorded, the controls helped to determine whether exposure to laser light for 60 seconds of a solution containing solely the uric acid or the sensitiser resulted in a concomitant absorbance shift. A final control solution was made up, its constituents being the same as L+U+S+ however this was not exposed to laser light (L-U+S+), the absorbance measurements for this solution were taken at the same time, thus determining whether any observed absorbance shift was laser light independent, the assay was then repeated.

3.2.5 Growth of planktonic oral bacteria

The organisms used were as follows:

- *Streptococcus sanguinis*
- *Porphyromonas gingivalis*
- *Fusobacterium nucleatum*
- *Actinobacillus actinomycetemcomitans*

The methods used to cultivate these microorganisms were the same as those described in chapter 2, section 2.1.1.

3.2.6 Susceptibility of planktonic oral bacteria to TBO/HeNe laser light

The method used was the same as that described in chapter 2, section 2.6.2 with the following exception:

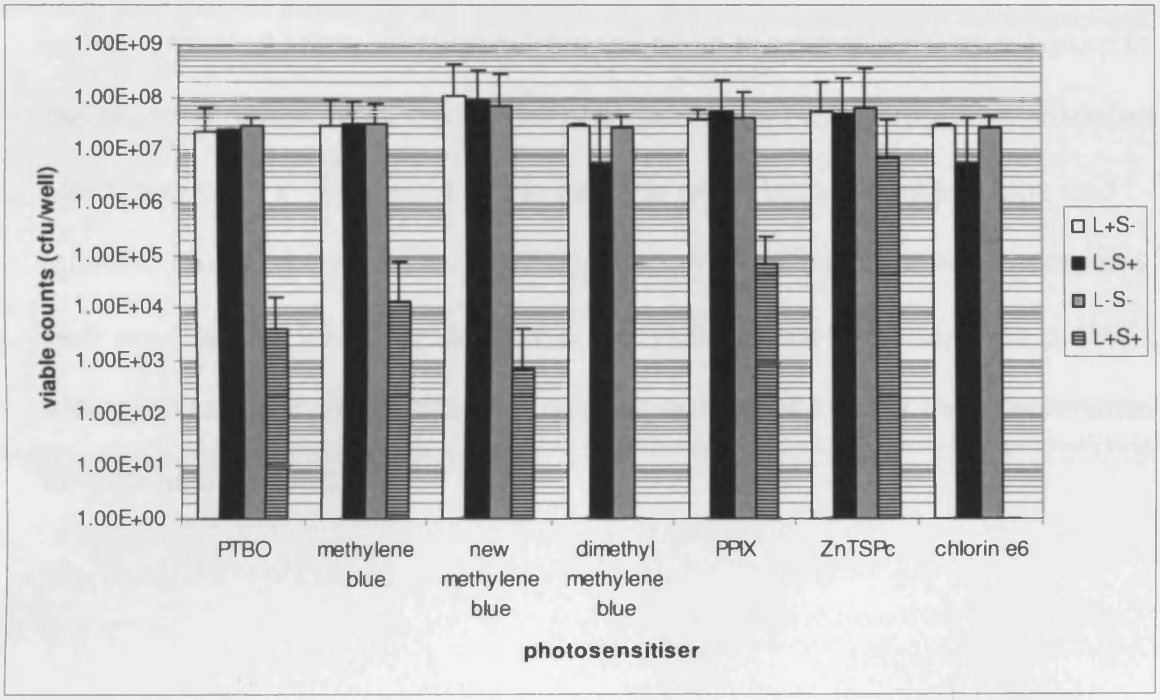
1. For each experiment the comparative efficacy of pharmaceutical grade TBO (pTBO, 99% pure) versus Sigma grade TBO (sTBO, 90% pure) was determined.

3.2.7 Statistical analysis of the data

The statistical significance of the data was ascertained using the two-tail t-test assuming unequal variance.

3.3 Results

Figure 3.1: Viable counts of *Streptococcus sanguinis* exposed to 2.1 J of HeNe laser light (energy density, 21.8 Jcm^{-2}) in the presence of 40.85 μM of various photosensitising compounds.



L+S+: Exposure to both laser light and photosensitiser

L-S- : Exposure to neither laser light nor photosensitiser

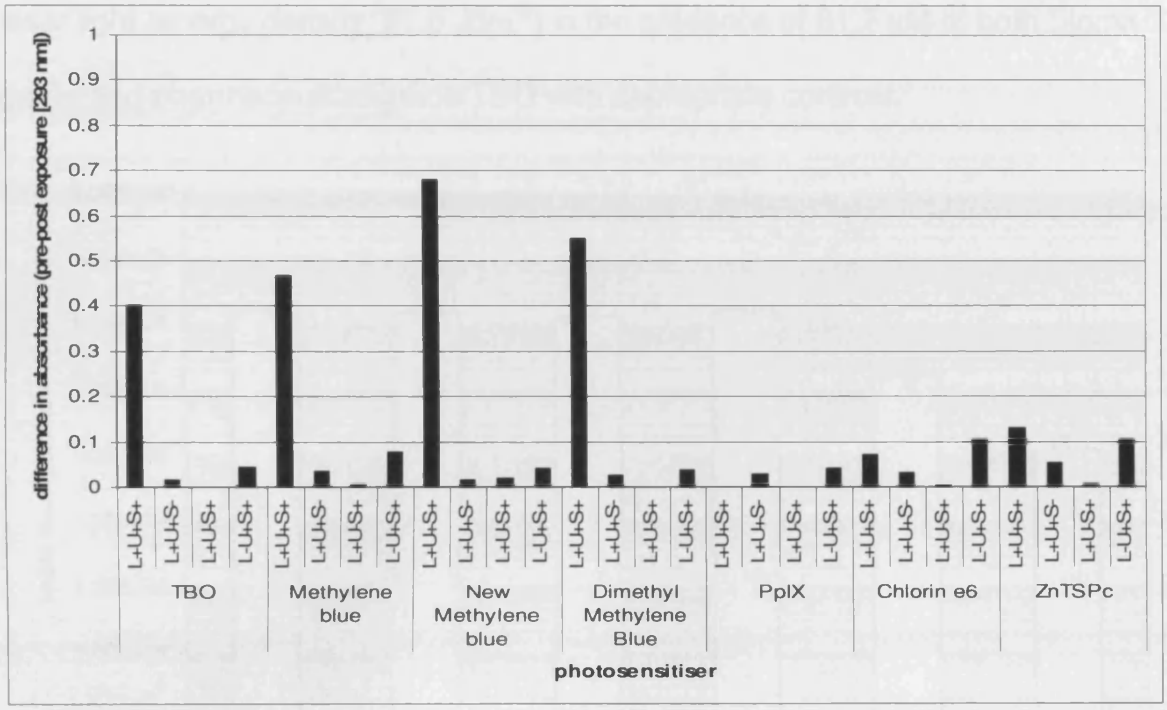
L+S-: Exposure to laser light but not to photosensitiser

L-S+: Exposure to photosensitiser but not to laser light

Exposure to laser light in the presence of the test photosensitisers resulted in a decrease in recovered viable bacteria in all instances as is shown in Figure 3.1. The photosensitiser that proved to be the least efficacious was zinc phthalocyaninetetrasulfonic acid where a reduction in bacterial numbers of less

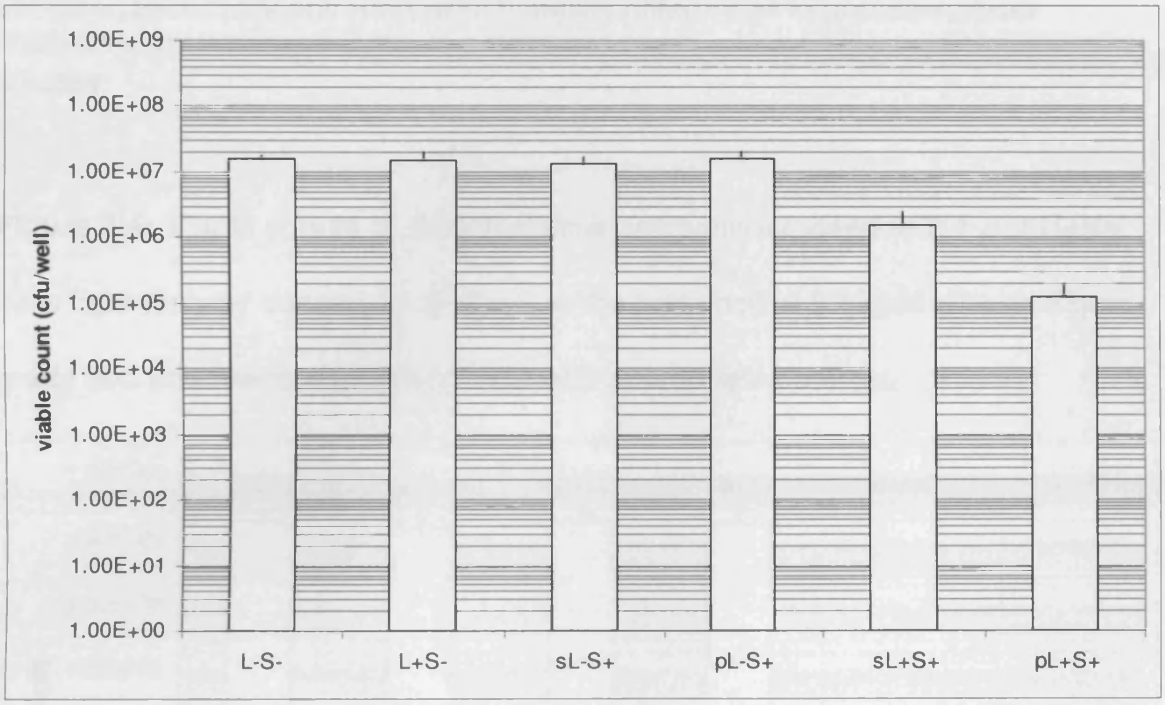
then 90 % was recorded. The greatest reductions were seen with the photosensitisers dimethyl methylene blue and chlorin e6, both of these resulted in an appreciable kill of the organism, no viable bacteria were recovered. However, these compounds exhibited dark toxicity, this is defined as a reduction in the number of recovered viable bacteria when the organism was exposed to the photosensitiser in the dark. The photosensitiser protoporphrin IX produced a >99% reduction in recovered viable bacteria whilst both methylene blue and toluidine blue O produced a > 99.9% reduction. Application of new methylene blue resulted in a 99.99% reduction in recovered viable bacteria, none of the aforementioned photosensitisers exhibited dark toxicity under the experimental conditions employed.

Figure 3.2: Relative singlet oxygen yield of the photosensitising compounds



Only four of the photosensitising compounds TBO, methylene blue, new methylene blue and dimethyl methylene blue upon illumination with HeNe laser light generated appreciable amounts of singlet oxygen, as is shown in Figure 3.2. The other photosensitising compounds assayed produced little singlet oxygen. The observed order of singlet oxygen yield was new methylene blue > dimethyl methylene blue > methylene blue > TBO.

Figure 3.3: Viable counts of *Streptococcus sanguinis* exposed to 2.1 J of HeNe laser light (energy density, 21.8 Jcm^{-2}) in the presence of 81.7 μM of both Sigma grade and pharmaceutical grade TBO with appropriate controls.



sL+S+: Exposure to both laser light and sigma grade TBO

pL+S+: Exposure to both laser light and pharmaceutical grade TBO

sL-S+: Exposure to sigma grade TBO but not to laser light

pL-S+: Exposure to pharmaceutical grade TBO but not to laser light

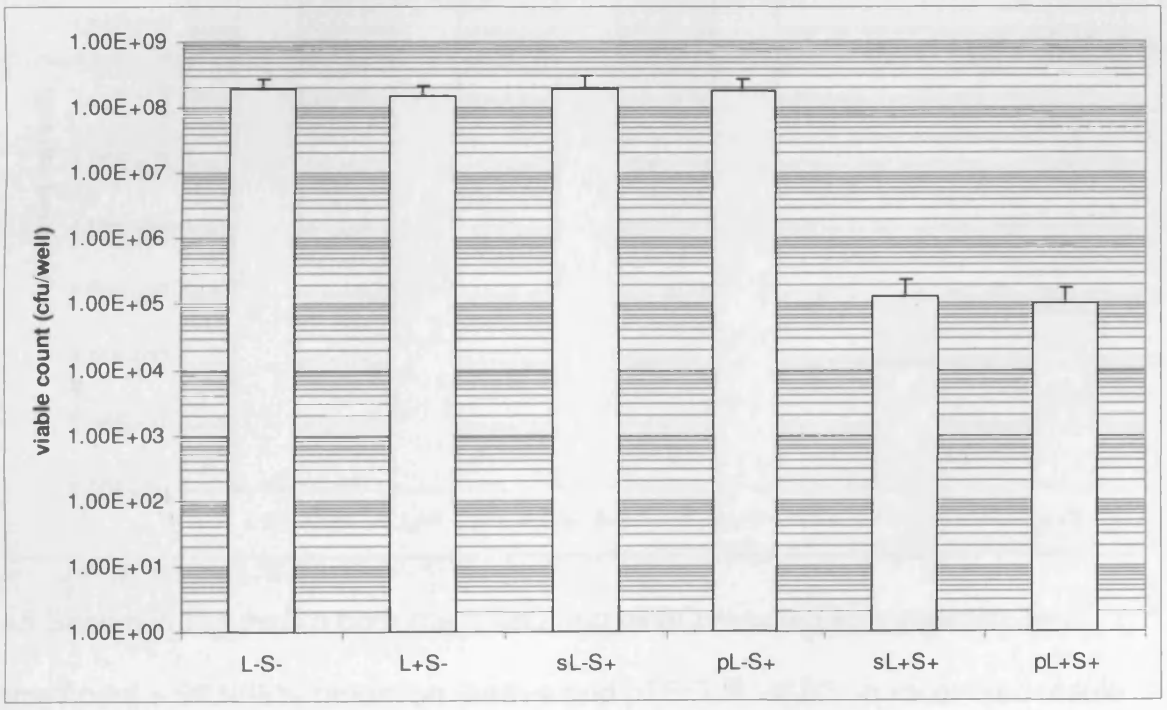
L-S- : Exposure to neither laser light nor TBO

L+S-: Exposure to laser light but not to TBO

Both the Sigma ($P < 0.01$) and pharmaceutical grade TBO ($P < 0.01$) upon illumination with the HeNe laser light produced a statistically significant reduction in recovered viable bacteria (L+S+) when compared to the control wells (L-S-) as is seen in Figure 3.3. However, use of the pTBO resulted in a significantly

greater reduction (P = 0.005) in bacterial numbers when compared to the sTBO. This experiment was modified, using a lower concentration of photosensitiser, to determine whether the same trend was still evident, and whether reducing photosensitiser concentration would amplify differences in photosensitiser efficacy.

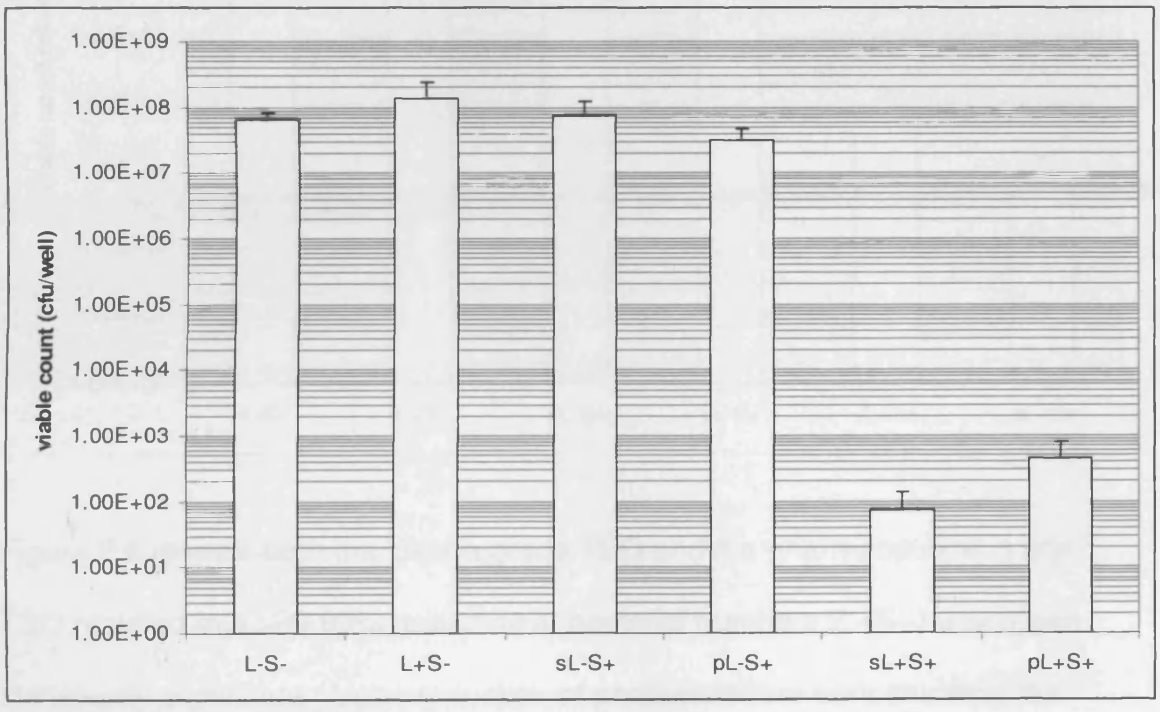
Figure 3.4: Viable counts of *Streptococcus sanguinis* exposed to 2.1 J of HeNe laser light (energy density, 21.8 Jcm^{-2}) in the presence of 8.17 μM of both Sigma grade and pharmaceutical grade TBO with appropriate controls.



Reducing the photosensitiser concentration ten-fold whilst maintaining all other experimental parameters resulted in both sTBO and pTBO producing a 99.9% reduction in recovered viable bacteria (L+S+) when compared to the control wells (L-S-) as is seen in Figure 3.4, this was found to be statistically significant (both s

and pTBO P <0.01). Use of a lower photosensitiser concentration revealed there was no difference in efficacy between the two grades of TBO.

Figure 3.5: Viable counts of *Porphyromonas gingivalis* exposed to 2.1 J of HeNe laser light (energy density, 21.8 Jcm^{-2}) in the presence of 81.7 μM of both Sigma grade and pharmaceutical grade TBO with appropriate controls.



As Shown in Figure 3.5 both the sTBO and pTBO resulted in a statistically significant > 99.999% reduction (both s and pTBO, P <0.01) in recovered viable bacteria (L+S+) of *Porphyromonas gingivalis* when compared to the control (L-S-), at a final concentration of 81.7μm. The experiment was then repeated at a lower sensitiser concentration.

Figure 3.6: Viable counts of *Porphyromonas gingivalis* exposed to 2.1 J of HeNe laser light (energy density, 21.8 Jcm^{-2}) in the presence of 8.17 μM of both Sigma grade and pharmaceutical grade TBO with appropriate controls.

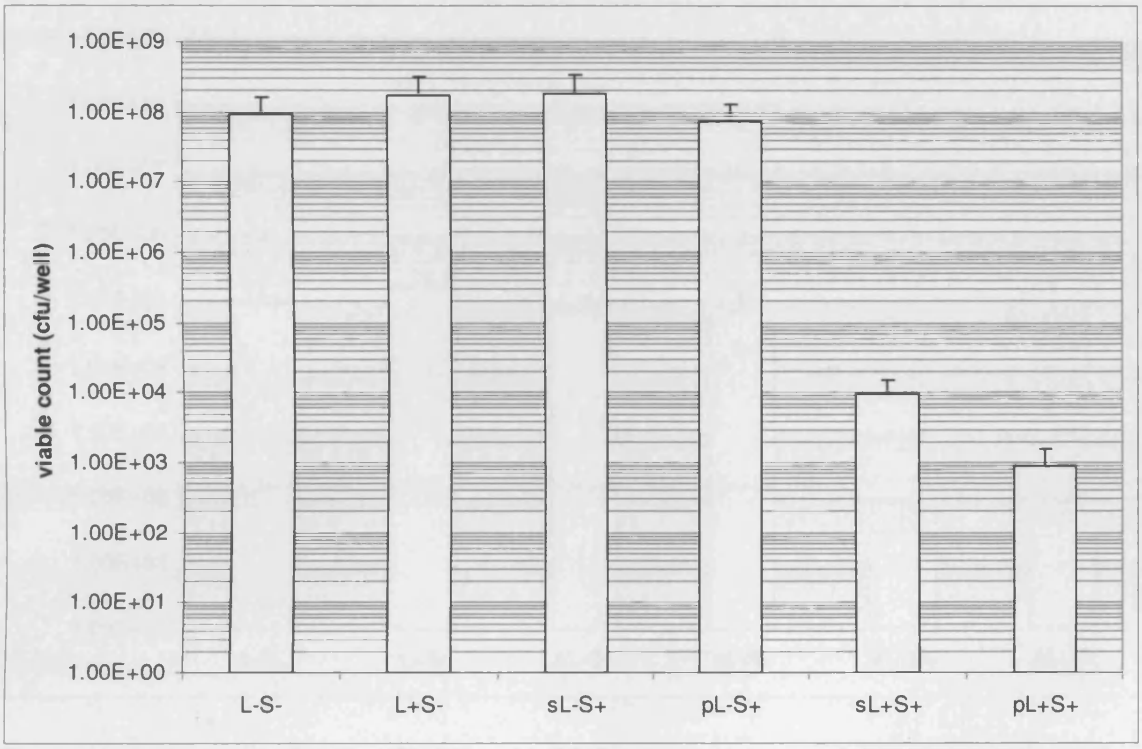


Figure 3.6 reveals both the Sigma grade TBO and the pharmaceutical grade TBO resulted in a >99.99% reduction in bacterial numbers (L+S+) which was statistically significant. Upon reduction of photosensitiser concentration the pharmaceutical grade TBO was found to be the most efficacious (pTBO P = 0.009027, sTBO P = 0.009031), this difference in efficacy was found to be statistically significant (P = 0.004).

Figure 3.7: Viable counts of *Fusobacterium nucleatum* exposed to 2.1 J of HeNe laser light (energy density, 21.8 Jcm^{-2}) in the presence of 81.7 μM of both Sigma grade and pharmaceutical grade TBO with appropriate controls.

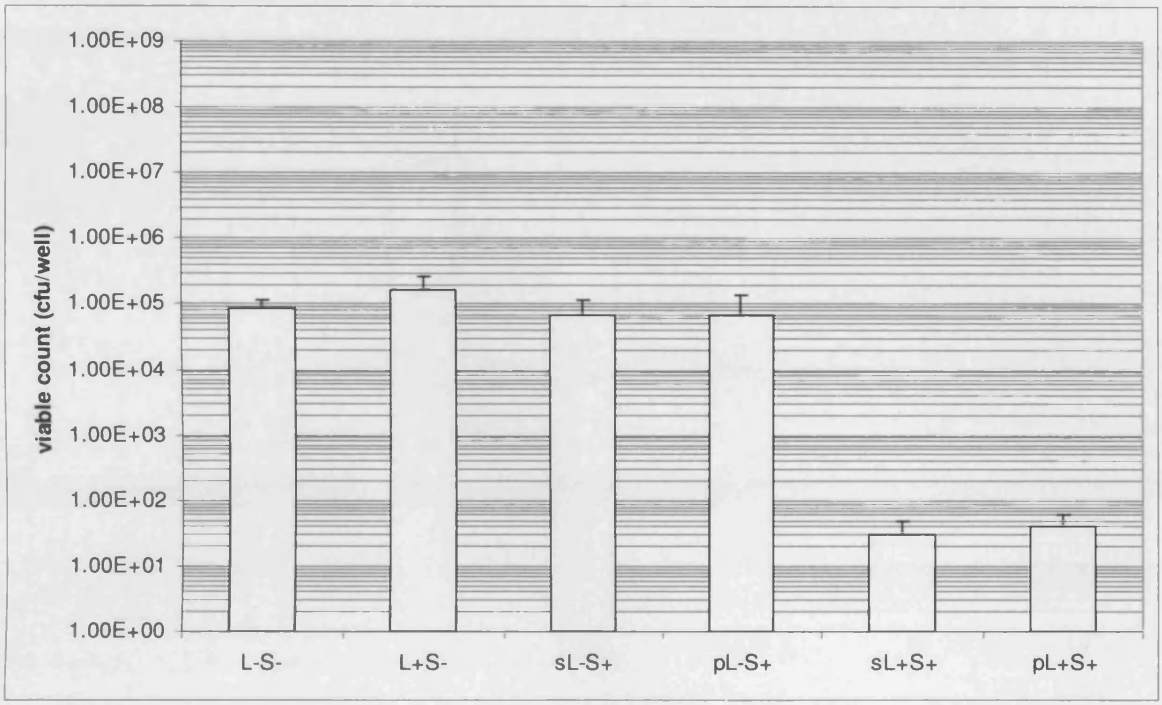
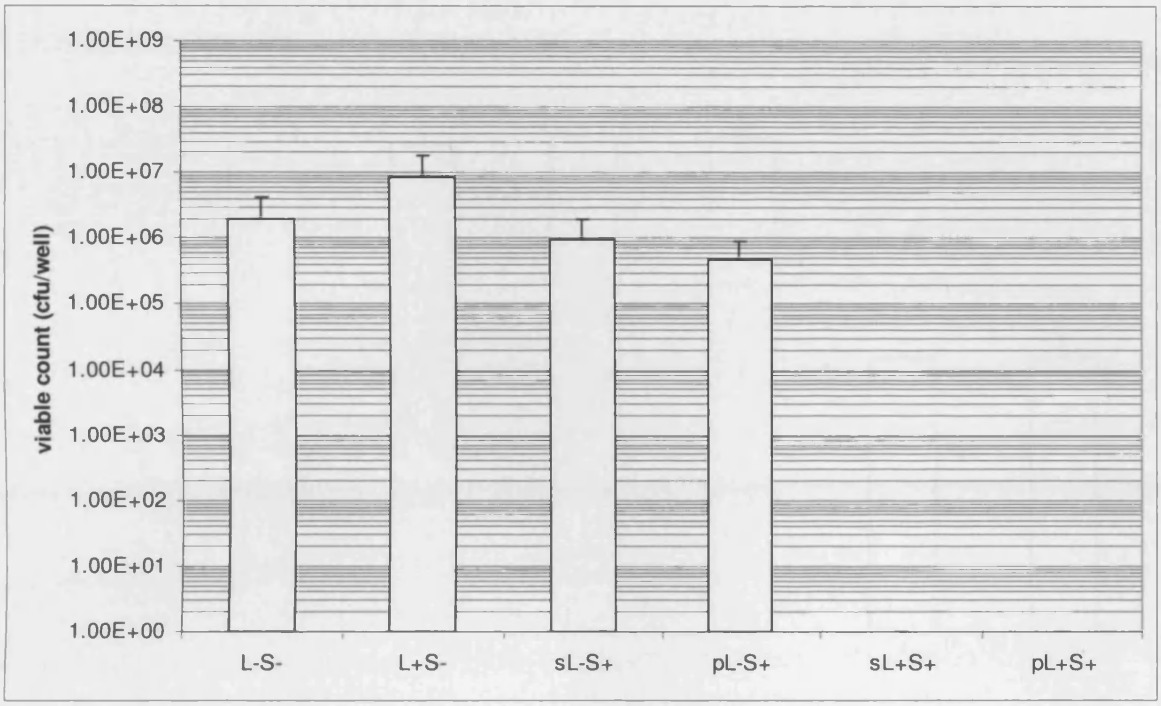


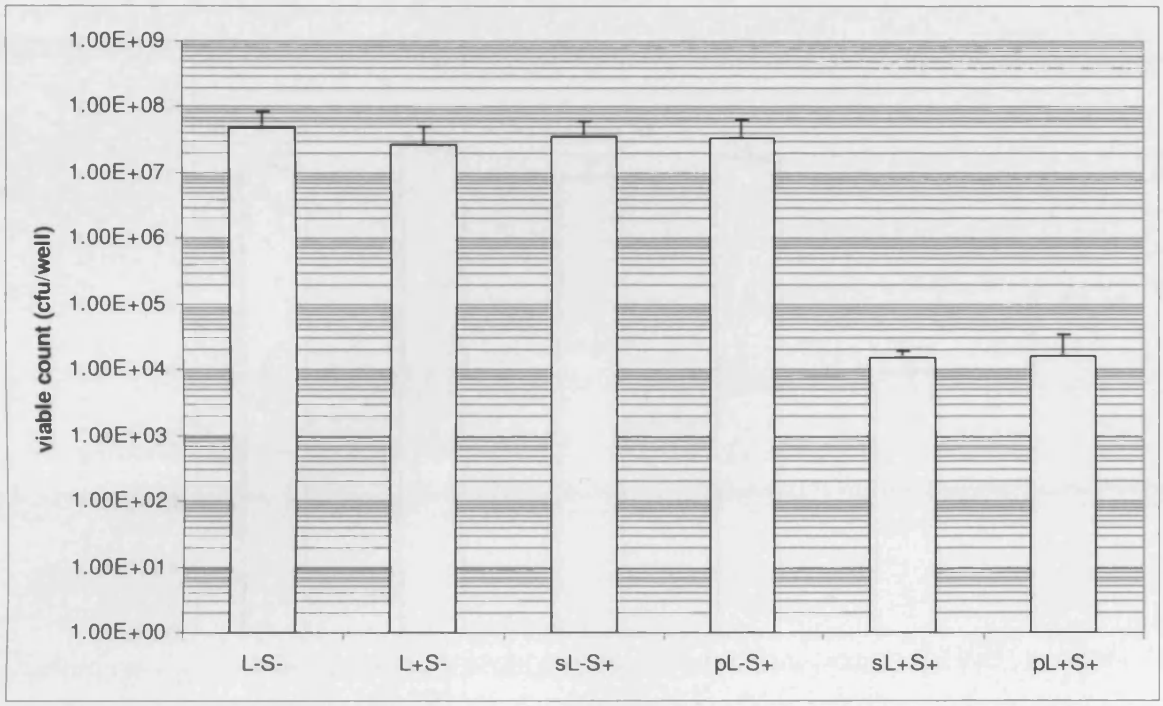
Figure 3.7 shows both the sTBO and pTBO produced a statistically significant > 99.9% reduction (both s and pTBO $P < 0.01$) in recovered viable bacteria (L+S+) when compared to the control wells (L-S-). The photosensitiser concentration was lowered whilst maintaining all other experimental parameters to amplify any differences in photosensitiser efficacy.

Figure 3.8: Viable counts of *Fusobacterium nucleatum* exposed to 2.1 J of HeNe laser light (energy density, 21.8 Jcm^{-2}) in the presence of 8.17 μM of both Sigma grade and pharmaceutical grade TBO with appropriate controls.



Both the sTBO and pTBO in conjunction with the HeNe laser light resulted in the total killing of the test organism as shown in Figure 3.8. The reduction in bacterial numbers elicited by both photosensitisers was >99.999%.

Figure 3.9: Viable counts of *Actinobacillus actinomycetemcomitans* exposed to 2.1 J of HeNe laser light (energy density, 21.8 Jcm^{-2}) in the presence of 81.7 μM of both Sigma grade and pharmaceutical grade TBO with appropriate controls.



Application of 81.7μM of both sTBO and pTBO as seen in figure 3.9 resulted in a >99.9% reduction in recovered viable bacteria (L+S+) of the test organism when compared to the control wells (L-S-), this was found to be statistically significant (both s and pTBO P = 0.01). Photosensitiser concentration was again reduced ten-fold to amplify any differences in efficacy.

Figure 3.10: Viable counts of *Actinobacillus actinomycetemcomitans* exposed to 2.1 J of HeNe laser light (energy density, 21.8 Jcm^{-2}) in the presence of 8.17 μM of both Sigma grade and pharmaceutical grade TBO with appropriate controls.

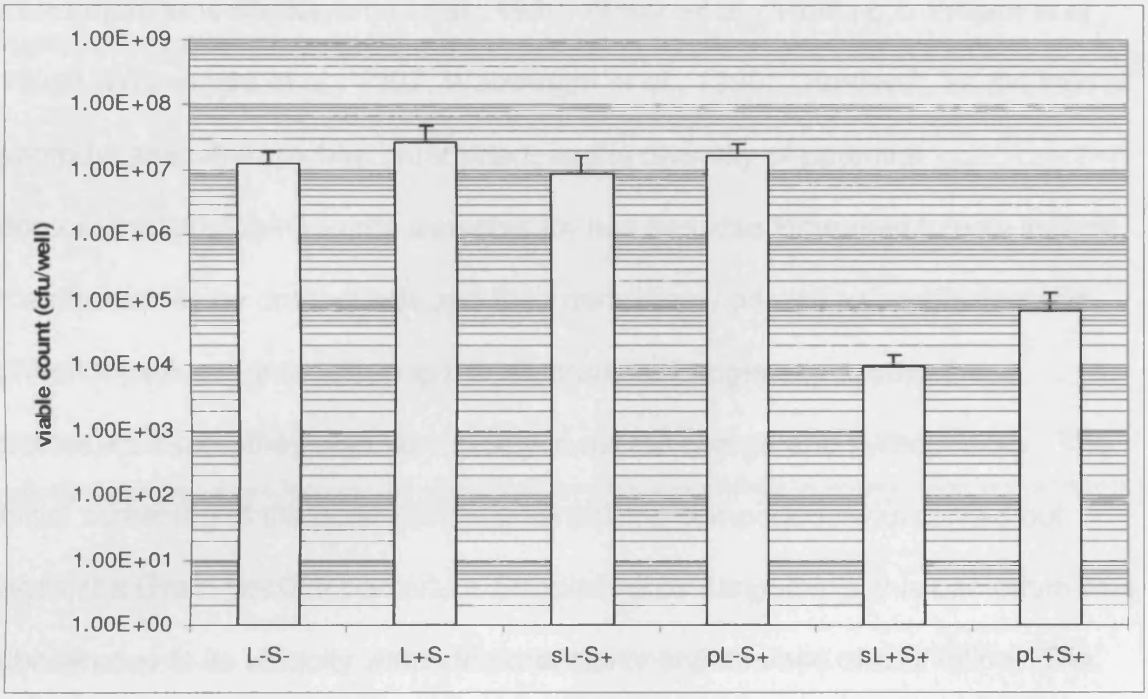


Figure 3.10 shows that reducing the photosensitiser concentration resulted in a statistically significant <99% reduction in bacterial numbers (both s and pTBO P = 0.002), in addition at the lower photosensitiser concentration the sTBO produced a somewhat greater kill than the pharmaceutical grade TBO, this observed difference was found to be statistically significant (P = 0.03). This apparent difference in efficacy was not evident at the higher concentration of photosensitiser used.

3.4 Discussion

As alluded to previously, the phenothiazine dyes have been shown to be efficacious photosensitising agents of both Gram-positive and Gram-negative microorganisms (Wakayama et al., 1980, Wilson et al., 1993a,b,c, Wilson et al., 1994b, Wainwright et al., 1997, Wainwright et al., 1999). However, as the field of photodynamic therapy has broadened, so the diversity of potential photosensitising compounds available for use has also increased to now include the phenothiazine compounds and their derivatives, as well as porphyrins and phthalocyanines. In addition to the structural heterogeneity among these photosensitisers, they also vary widely in overall charge and hydrophilicity. The initial screening of the potential photosensitising compounds was carried out against a Gram-positive bacterium *Streptococcus sanguininis,* this bacterium was chosen due to its ubiquity within the oral cavity and its ease of cultivation. The results showed that several of the test compounds proved to be efficacious photosensitising agents of the organism, among them chlorin e6. However, this compound demonstrated considerable dark toxicity, an attribute not desirable in a potential photosensitiser. This consequently led to it being discounted as a potential photobactericidal agent for the purposes of this study. Of the remaining compounds, all possessed differing photosensitising capabilities, the phenothiazine compounds proved to be the most promising group of photobactericidal compounds tested.

In addition to lethal photosensitisation, a high singlet oxygen yield is often indicative of an efficient photosensitiser, and it is hypothesised that it is the primary agent responsible for photoinactivation via the type II pathway (Valduga et al., 1993), although the importance of type I reactions remains unclear (Martin et al., 1987a,b, Burch., 1989). The phenothiazines MB, TBO, NMB and DMMB all proved to be efficacious photobactericidal agents of *Streptococcus sanguinis* and yielded significant amounts of singlet oxygen when assayed. DMMB has previously been employed as a photosensitiser against various strains of MRSA (Wainwright et al., 1998a), interestingly when the compound was assayed against a wider range of bacterial species DMMB did not exhibit enhanced bactericidal activity upon illumination, evidence of dark toxicity (Wainwright et al., 1997). The primary advantage of photodynamic therapy for the treatment of bacterial infections is the inherent specificity of photobactericidal agents; the bactericidal action of the compound may be limited as cytotoxicity is due to the combined action of photosensitiser and laser light. Thus any potential perturbation of the microbiota is precluded at adjoining sites where solely photosensitiser may be present. This is particularly important within the oral cavity as the commensal microbiota prevent colonisation by other, potentially pathogenic species. The spectre of dark toxicity consequently led to DMMB being discounted as a potential photobactericidal agent for this study

The photosensitiser zinc phthalocyaninetetrasulfonic acid was not particularly effective as a photobactericidal agent, possibly as the laser utilised generated

light of a wavelength 632.8 nm whereas this compound absorbs maximally at 675 nm this may also explain the low observed quantum yield of singlet oxygen. This class of compound had previously been demonstrated to be an efficacious photobactericidal compound (Wilson et al.,1994c, Wilson et al.,1995a, Griffiths et al.,1997) when allied with an appropriate source of light.

The photosensitiser protoporphyrin IX when used against *S. sanguinis* resulted in a >99% reduction in the bacterial count, however when assayed for singlet oxygen production it was found to have a poor quantum yield. A low quantum yield of singlet oxygen indicates that this compound may be a poor photosensitiser. This suspicion was confirmed when the photobactericidal activity was examined, compared to other compounds assayed, notably the phenothiazines, this compound was not as efficacious a photosensitiser. It should also be noted that the negatively-charged porphyrin class of photosensitiser are reported to be ineffective sensitising agents of Gram-negative bacteria, this may be overcome via conjugating to, or addition with, a cationic agent (Nitzan et al.,1992, Merchat et al.,1996, Rovaldi et al.,2000), whilst the organism used in the initial study was not Gram-negative, many putative periodontal pathogens are Gram-negative.

MB and TBO are known to be efficacious light activated antimicrobials, previous findings demonstrated that both TBO and MB exert photobactericidal activity against a range of microorganisms (Usacheva et al.,2001). The results obtained

in the course of this study show both TBO and MB to be capable of photosensitising *S. sanguinis*. NMB exhibited a potent antibacterial action when illuminated and produced a higher quantum yield of singlet oxygen than either TBO or MB, the results confirm NMB to be an extremely effective photosensitiser. Unlike both TBO and MB, NMB has not been as thoroughly investigated, the cursory nature of the initial screening process meant that important photophysical properties of the compound, such as potential mammalian toxicity remain unknown (an important aspect if the agent is to have any therapeutic value), rendering the compound unsuitable as a potential photosensitiser for the purposes of this study. Hence, due to its high quantum yield of singlet oxygen and efficacy in killing large numbers of *Streptococcus sanguinis,* TBO was the chosen photosensitiser for further investigation. Although MB produced a higher quantum yield of singlet oxygen it was not as an efficacious photobactericidal agent as TBO, whilst singlet oxygen yield is important, it only alludes to potential photosensitisng ability. Moreoever, TBO is known to be an effective sensitising agent of both cariogenic and periodontopathogenic organisms in conjunction with a HeNe light source (the light source available for the duration of this study) many of these organisms are known constituents of oral biofilms, the lethal photosensitisation of which being one of the aims of this investigation.

Two grades of TBO were available for use throughout this study, pharmaceutical grade TBO (pTBO) which is approximately 99% pure and TBO purchased from

Sigma Chemicals (sTBO) which is c. 90% pureBoth were used in subsequent experiments against planktonic oral organisms to determine what effect, if any, photosensitiser purity had on the photobactericidal effect of TBO. The efficacy of both grades of photosensitiser varied depending on the target organism. In an effort to optimise the investigation, the concentration of photosensitiser was reduced to amplify any differences in efficacy between the two grades. Interestingly the effect of reducing photosensitiser concentration varied from organism to organism. Reducing photosensitiser concentration tenfold resulted in a greater photobactericidal effect with respect to *S. sanguinis* and *F. nucleatum,* indeed in the case of *F. nucleatum* reducing concentration resulted in no viable colonies being present post illumination; whereas with *P. gingivalis* and *A. actinomycetemcomitans* reducing photosensitiser concentration did not result in concomitant increased photobactericidal effect. This difference in efficacy was intriguing, *S. sanguinis* is a Gram-positive bacterium, whilst *F. nucleatum* is Gram-negative upon staining, but is phylogentically classified as a Gram-positive whereas *P. gingivalis* and *A. actinomycetemcomitans* are Gram-negative. It has previously been mooted that Gram-negative organisms may prove to be recalcitrant to photosensitisation due to the charge of the photosensitiser, neutral or negatively-charged compounds tend to be ineffective (Minnock et al., 1996); this is unlikely in this instance as TBO is a cationic photosensitiser. It has also been hypothesised that Gram-negative bacteria possess increased resistance to photosensitisation due to the barrier function of the outer membrane, which impedes access of any generated free radicals to the cytoplasmic membrane

(Nitzan et al., 1992). It has been postulated that photosensitisation of Gram-negative bacteria requires disturbance of the cytoplasmic membrane (Nitzan et al., 1992). The data presented suggests that there is no consistent difference in efficacy between the two grades of TBO in relation to the photosensitisation of planktonic organisms, this however may not apply to biofilm-cultivated organisms.

The results of the experiments contained in this chapter demonstrate that the phenothiazines, especially NMB, MB and TBO are efficacious photosensitising agents of *S. sanguinis,* and when assayed all produced significant quantities of singlet oxygen. TBO was the photosensitiser chosen for use in the remainder of the study, due to its efficacy against *S. sanguinis* and high yield of singlet oxygen. Upon testing of the two grades of TBO against both Gram-positive and Gram-negative planktonic oral organisms there was no consistent difference in efficacy, hence the investigation proceeded to determine the efficacy of TBO against oral organisms when grown as biofilms.

CHAPTER FOUR

LETHAL PHOTOSENSITISATION OF MEMBRANE GROWN MONO-SPECIES BIOFILMS

4.1 Introduction

It was established in the previous chapter that both Sigma and pharmaceutical grades of TBO were effective photosensitising agents of a range of Gram positive and Gram negative planktonic oral organisms. However if this photosensitiser was to be an effective photosensitiser *in vivo* it is essential that it was able to kill these organisms when they are present as biofilms. Therefore the experiments described within this chapter were to determine whether TBO (both the Sigma and pharmaceutical grade) were able to sensitise *S. sanguinis* and *A. viscosus*, both commensal organisms, and the putative pathogens *P. gingivalis* and *F. nucleatum*, when these organisms were in the form of mono-species biofilms. The large number of organisms being tested and the numerous experimental parameters necessitated a very simple biofilm model to facilitate this initial screening process. In addition CLSM was used to study both pristine biofilms and those which had previously been photosensitised, permitting the spatial distribution of both viable and non-viable bacteria to be mapped.

4.2 Materials and methods

4.2.1 Growth of planktonic oral bacteria

The organisms used were as follows:

- *Streptococcus sanguinis*
- *Porphyromonas gingivalis*
- *Fusobacterium nucleatum*
- *Actinomyces viscosus*

The method used was the same as that described in chapter 2, section 2.1.1., with the following exception:

The organism used was *Streptococcus sanguinis* NCTC 7863

4.2.2 Growth of biofilms on membrane filters

The method used to cultivate the mono-species biofilms was the same as that described in chapter 2, section 2.4

4.2.3 Preparation of TBO

This was carried out as described in chapter 2, section 2.6.1.1

4.2.4 Susceptibility of membrane grown biofilms to TBO/HeNe laser light

The method used was the same as that described in chapter 2, section 2.6.3. The resultant data was statistically analysed as described in section 4.2.6.

4.2.5 CLSM of membrane filter biofilms and subsequent analysis

The method used was the same as that described in chapter 2, section 2.5.

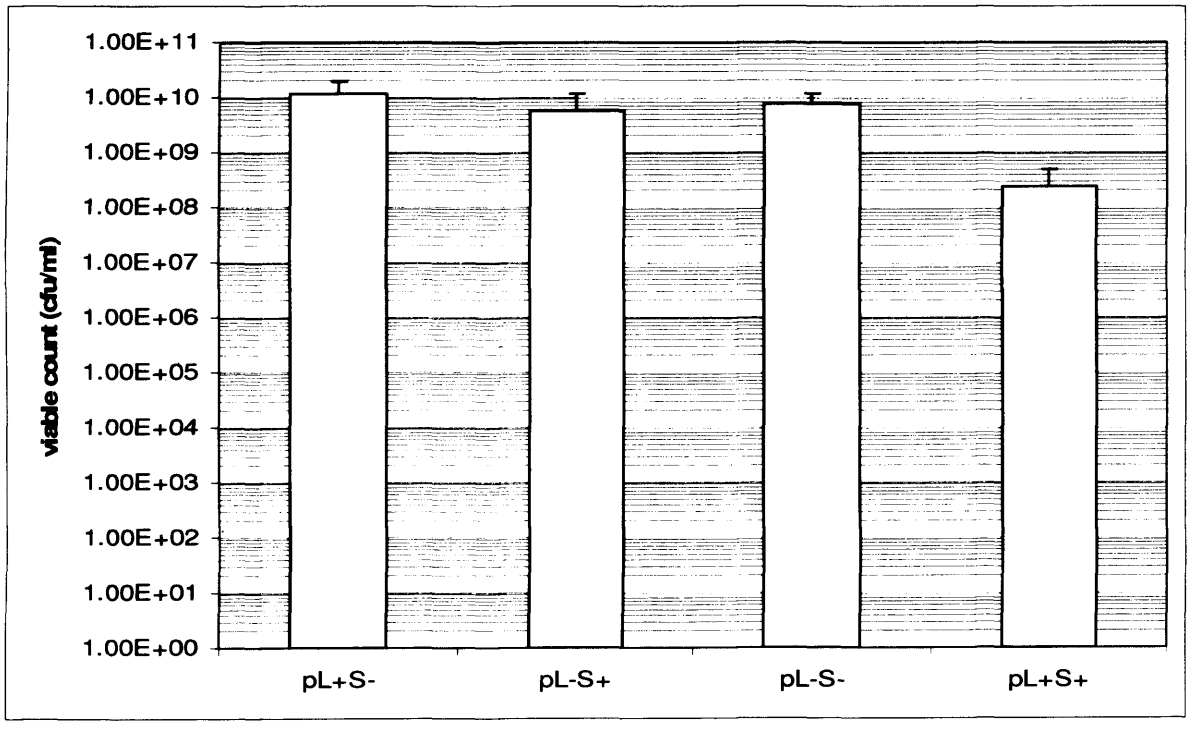
4.2.6 Statistical analysis of the data

The statistical significance of the data was ascertained using the two-tail t-test assuming unequal variance.

4.3 Results

Mono-species biofilms were generated using the membrane filter methodology, the biofilms were then subjected to lethal photosensitisation using either pharmaceutical or Sigma grade TBO in conjunction with HeNe laser light. CLSM was also performed on both photosensitised biofilms and those in their native state.

Figure 4.1: Viable counts of *Streptococcus sanguinis* biofilms cultivated on membrane filters exposed to 31.5 J of HeNe laser light (energy density, 81.9 Jcm^{-2}) in the presence of 10 µl of 81.7µM pTBO with appropriate controls.



pL+S+: Exposure to both laser light and pharmaceutical grade photosensitiser

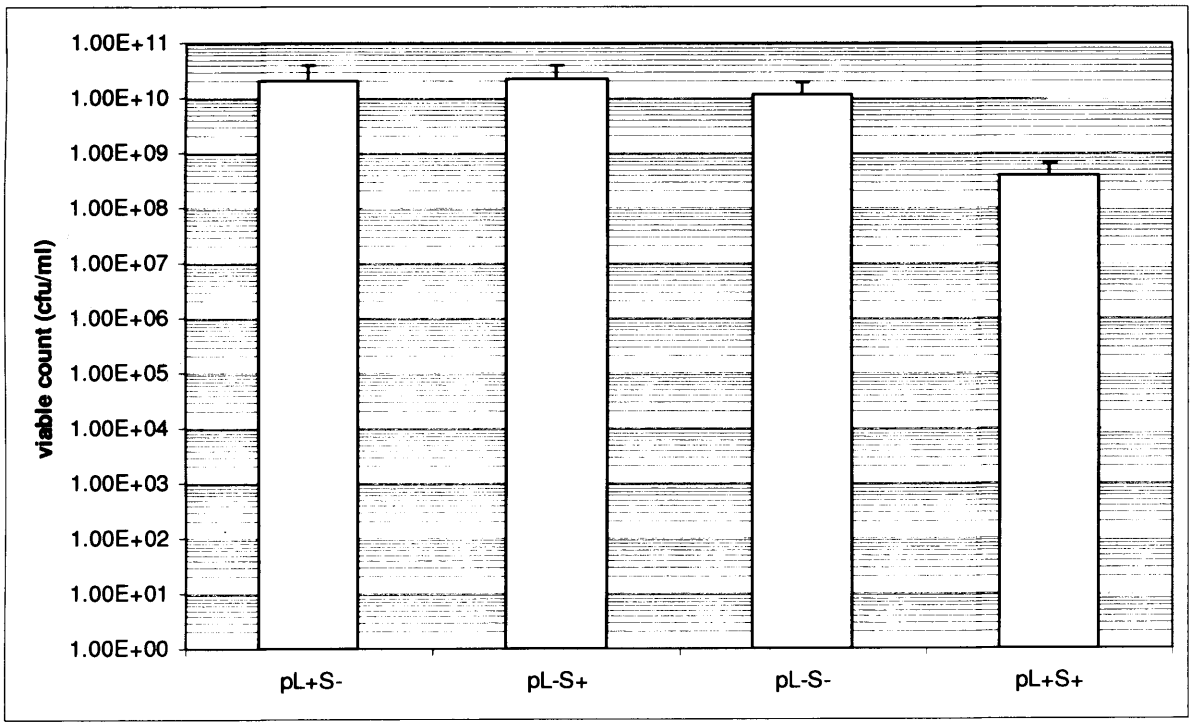
pL-S- : Exposure to neither laser light nor pharmaceutical grade photosensitiser

pL+S-: Exposure to laser light but not to pharmaceutical grade photosensitiser

pL-S+: Exposure to pharmaceutical grade photosensitiser but not to laser light

As can be seen in figure 4.1 exposure of *S. sanguinis* biofilms to pTBO in conjunction with the HeNe laser light resulted in a >90% reduction in recovered viable bacteria (L+S+) when compared to the control wells (L-S-), this reduction was found to be statistically significant (P = 0.0009).

Figure 4.2: Viable counts of *Porphyromonas gingivalis* biofilms cultivated on membrane filters exposed to 31.5 J of HeNe laser light (energy density, 81.9 Jcm^{-2}) in the presence of 10 µl of 81.7µM pTBO with appropriate controls.



As is evident from figure 4.2 use of pTBO in conjunction with the HeNe laser light resulted in a statistically significant (P = 0.006) >90% reduction in the viable

count (L+S+) when compared to the control wells (L-S-) of the *P.gingivalis* biofilms.

Figure 4.3: Viable counts of *Fusobacterium nucleatum* biofilms cultivated on membrane filters exposed to 31.5 J of HeNe laser light (energy density, 81.9 Jcm^{-2}) in the presence of 10 µl of 81.7µM pTBO with appropriate controls.

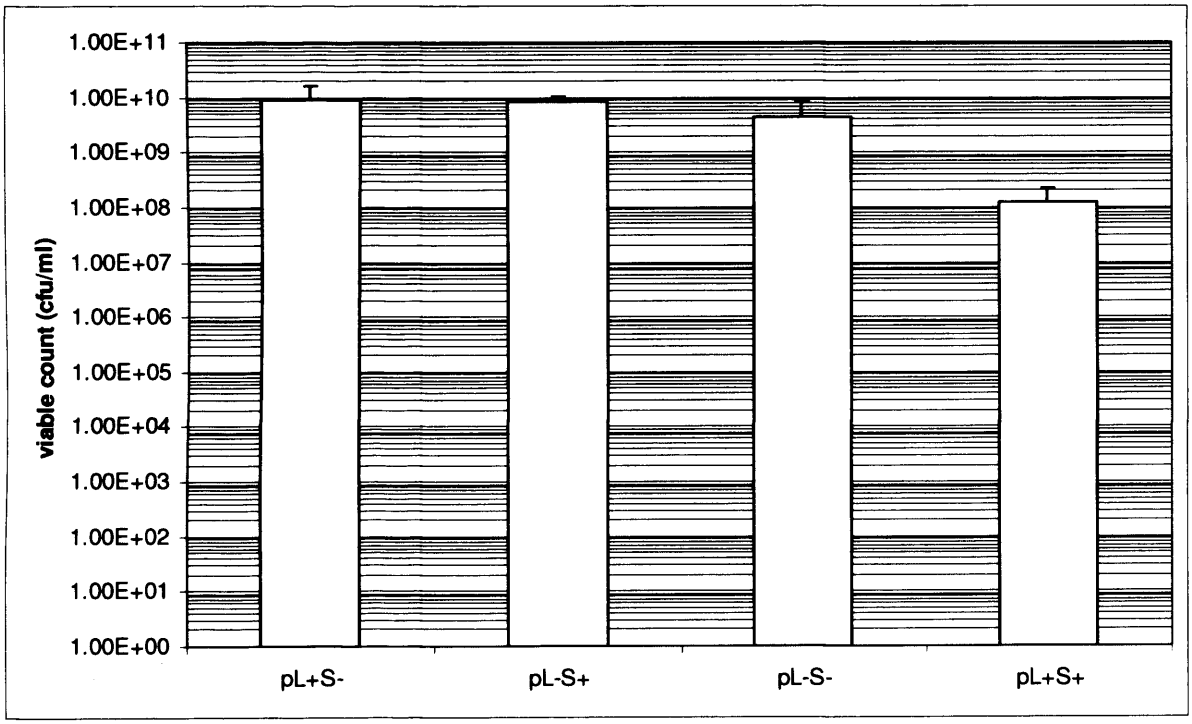


Figure 4.3 shows that pTBO in conjunction with HeNe laser light resulted in a statistically significant (P = 0.03) >90% reduction in recovered viable bacteria (L+S+) when compared to the control wells (L-S-).

Figure 4.4: Viable counts of *Actinomyces viscosus* biofilms cultivated on membrane filters exposed to 31.5 J of HeNe laser light (energy density, 81.9 Jcm^{-2}) in the presence of 10 µl of 81.7µM pTBO with appropriate controls.

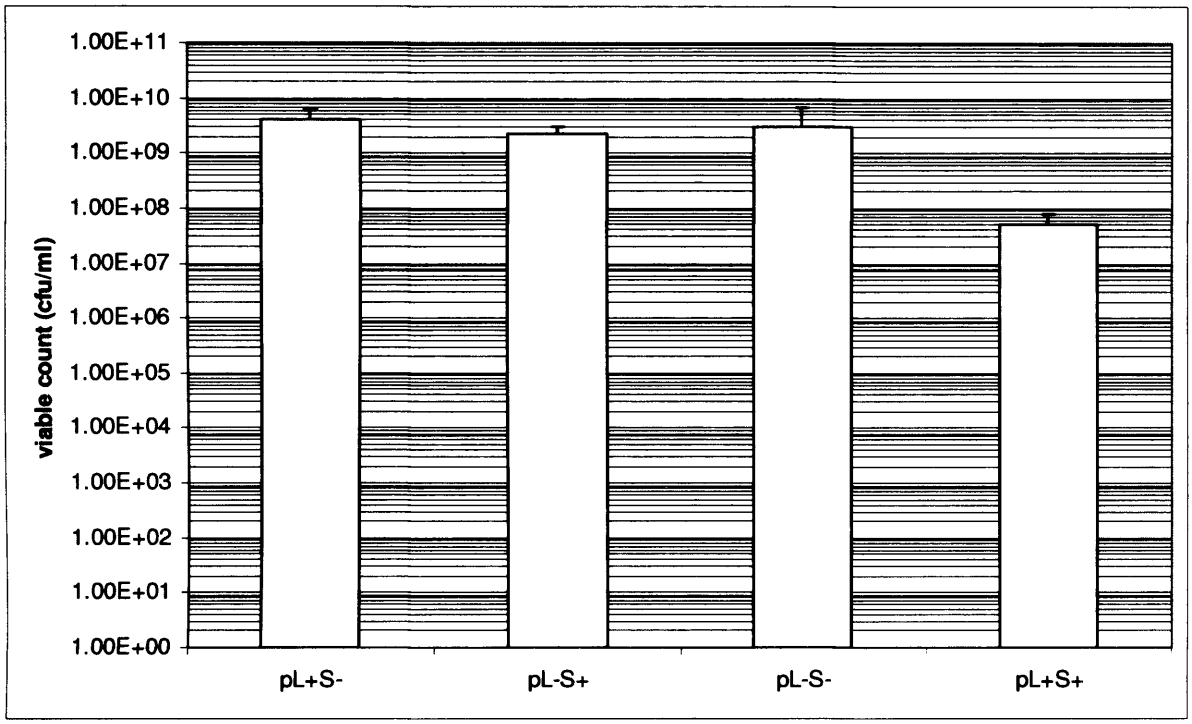
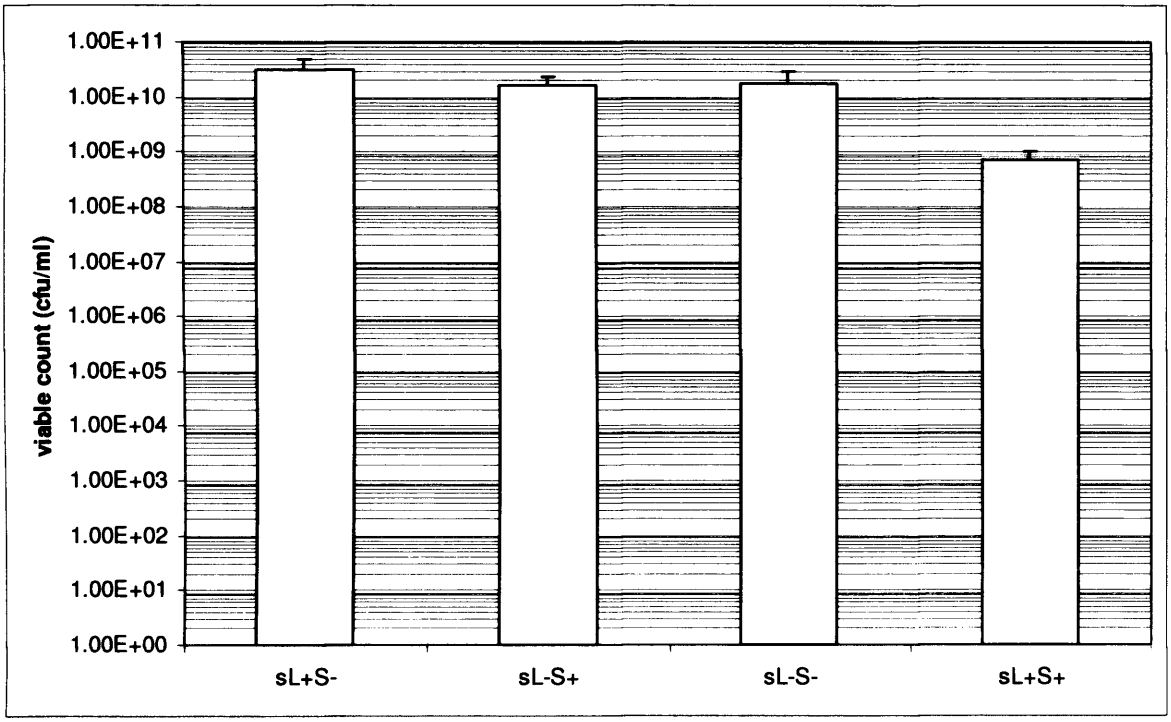


Figure 4.3 shows that pTBO in conjunction with HeNe laser light resulted in a statistically significant (P = 0.05) >90% reduction in recovered viable bacteria (L+S+) when compared to the control wells (L-S-). The reduction in the number of recovered viable organisms evident here was consistent with that obtained under identical experimental conditions with biofilms of *S. sanguinis*, *P. gingivalis* and *F. nucleatum*.

Figure 4.5: Viable counts of *Porphyromonas gingivalis* biofilms cultivated on membrane filters exposed to 31.5 J of HeNe laser light (energy density, 81.9 Jcm^{-2}) in the presence of 10 µl of 81.7µM sTBO with appropriate controls.



sL+S+: Exposure to both laser light and Sigma grade photosensitiser

sL-S- : Exposure to neither laser light nor Sigma grade photosensitiser

sL+S-: Exposure to laser light but not to Sigma grade photosensitiser

sL-S+: Exposure to Sigma grade photosensitiser but not to laser light

Figure 4.5 shows the substitution of pTBO with sTBO, this resulted in a statistically significant (P = 0.003) reduction of >90% in recovered viable bacteria (L+S+) when compared to the control wells (L-S-). This reduction was consistent with what was found when using pTBO.

Figure 4.6: Viable counts of *Fusobacterium nucleatum* biofilms cultivated on membrane filters exposed to 31.5 J of HeNe laser light (energy density, 81.9 Jcm^{-2}) in the presence of 10 µl of 81.7µM sTBO with appropriate controls.

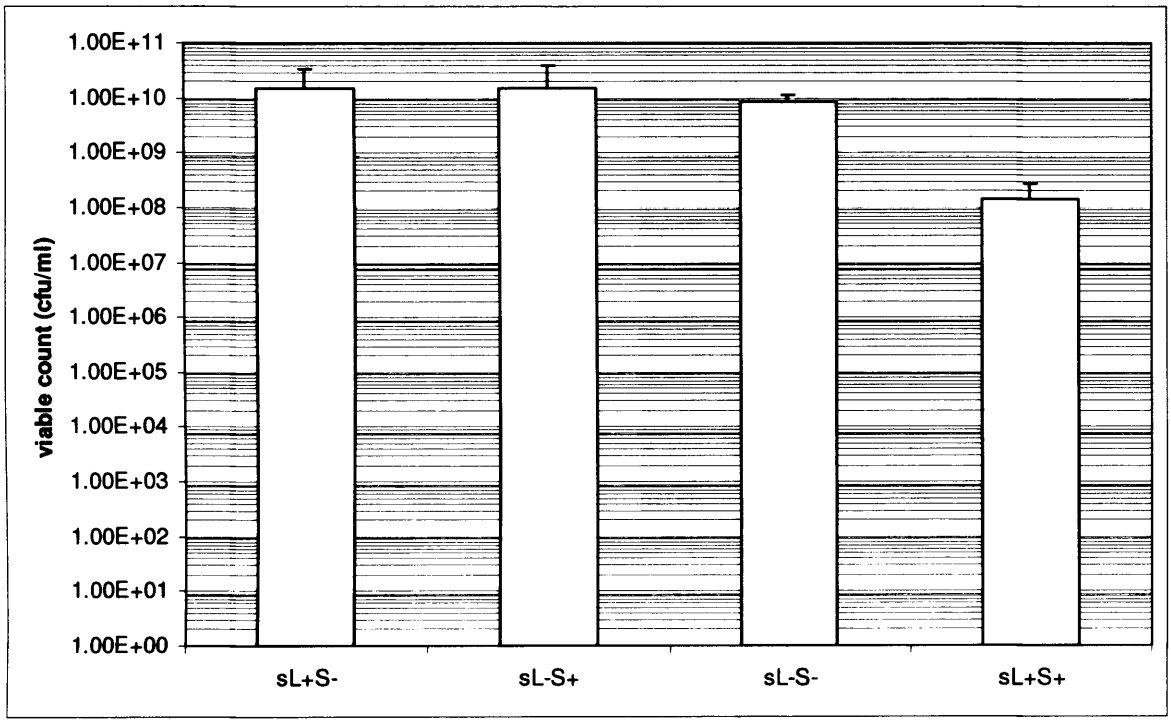


Figure 4.6 again showed that the substitution of pTBO with sTBO resulted in a statistically significant (P = 0.00007) reduction of >90% in recovered viable bacteria (L+S+) when compared to the control wells (L-S-), this was comparable with what was found when using the pTBO. No difference in efficacy was evident between the two grades of TBO when used against mono-species biofilms, the kills obtained being identical regardless of the grade of TBO used.

Figure 4.7a: a confocal micrograph of a control (L-S-) *P. gingivalis* biofilm. The bar represents 20 μm

There was a dramatic visual difference between the untreated control biofilm, figure 4.7a and the photosensitised biofilm, figure 4.7b, qualitatively there appeared to be a greater number of non-viable (stained red) than viable cells (stained green) present within the photosensitised biofilm, this contrasted with the control biofilm which appeared to show the reverse, within the control biofilm the architecture appeared to be intact, one could distinguish microcolony formations typical of biofilms.

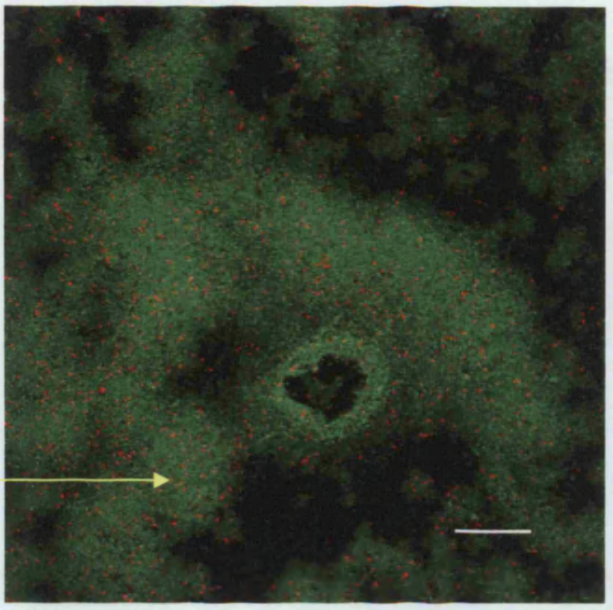


Figure 4.7b: a confocal micrograph of a *P. gingivalis* biofilm having been exposed to 31.5 J of laser light (energy density, 81.9 Jcm^{-2}) in the presence of 10 μl of 81.7μM pTBO. The bar represents 20 μm

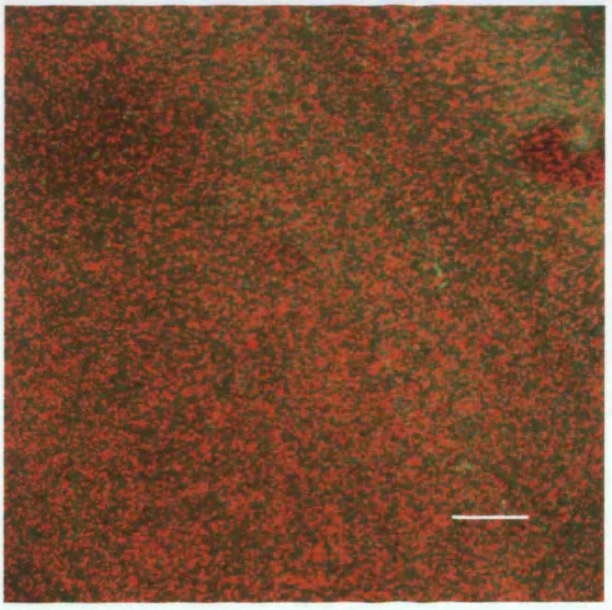
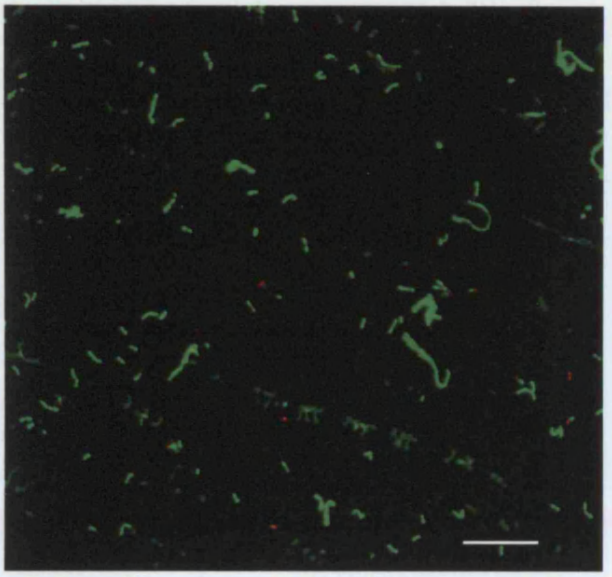


Figure 4.8a: a confocal micrograph of a control (L-S-) *S. sanguinis* biofilm. The bar represents 20 µm



When comparing figure 4.8a of the untreated (L-S-) biofilm and figure 4.8b which was subjected to lethal photosensitisation, qualitatively there appeared to be far higher proportions of nonviable cells apparent in the photosensitisied biofilm as compared to the control biofilm. Streptococcal chains were clearly identifiable, with single cells distinguishable in the photosensitised biofilm, many of the chains stained red indicating that a higher proportion of the cells were nonviable.

Figure 4.8b: a confocal micrograph of a S. sanguinis biofilm having been exposed to 31.5 J of laser light (energy density, 81.9 Jcm^{-2}) in the presence of 10 µl of 81.7µM pTBO. The bar represents 20 µm.

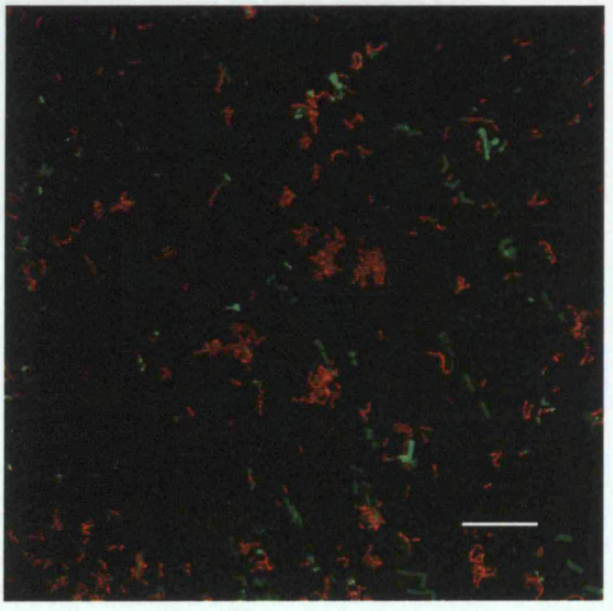


Figure 4.8c: The depth into a control (L-S-) *S. sanguinis* biofilm as determined by CLSM versus the normalised image intensity for both the viable and nonviable channels.

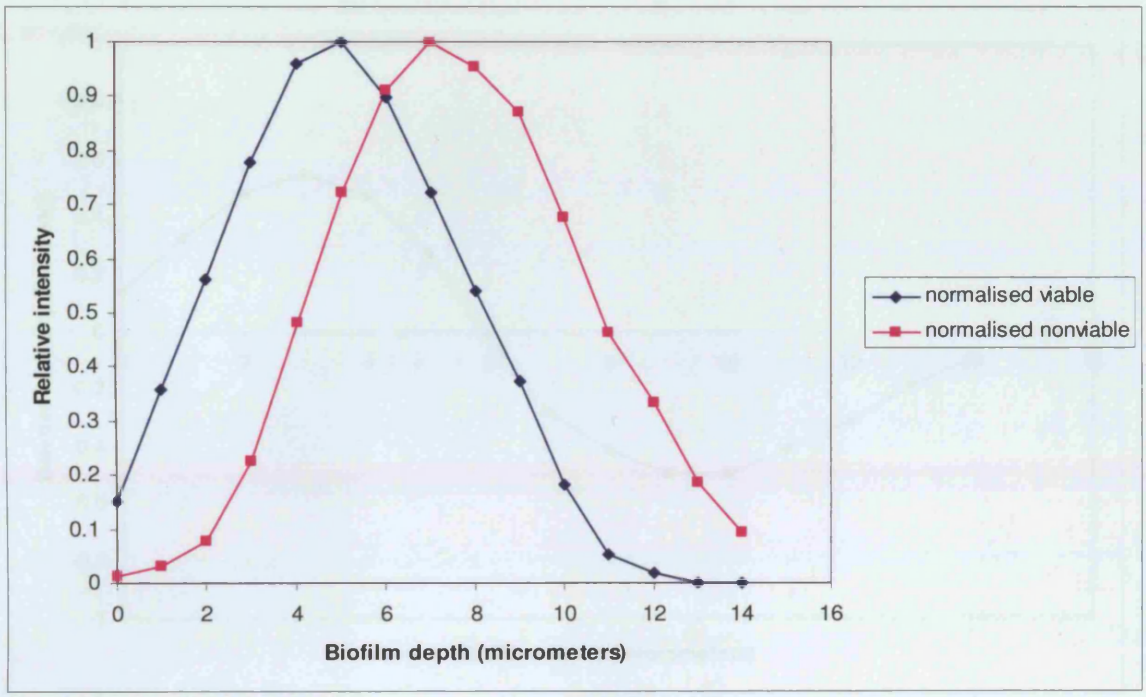


Figure 4.8c shows the relative image intensity, which has been normalised against the maximum image intensity for each channel for both the viable and nonviable cells. Image intensity within the untreated biofilm followed a 'normal distribution' with the relative intensity for both channels being at their lowest on the periphery of the biofilm. The peaks of the intensity distribution curves for both the viable and non viable channels were offset, indicating a higher preponderance of viable cells were in the upper portion of the biofilm declining on progression through the biofilm.

Figure 4.8d: The changes in the viable to nonviable relative image intensity (using normalised data) as a function of biofilm depth in a control (L-S-) *S. sanguinis* biofilm as determined by CLSM.

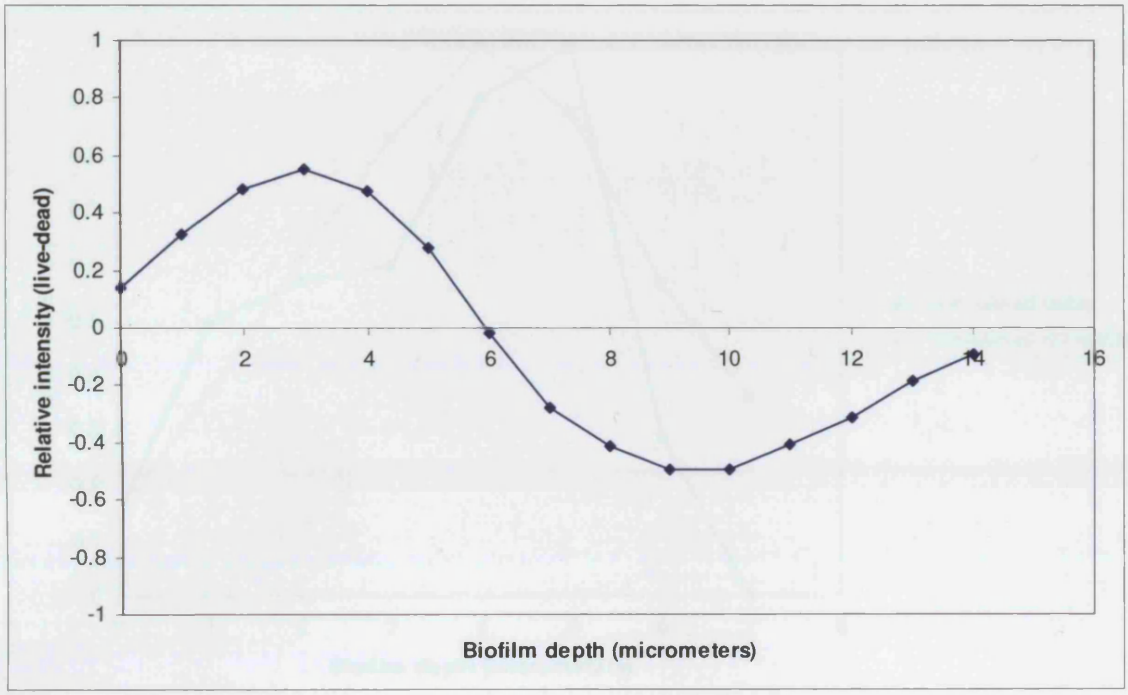


Figure 4.8d shows the relative intensity throughout the untreated (L-S-) biofilm. The relative intensity is a quantitative value, being a measure of the ratio of viable to nonviable cells (normalized nonviable intensity value subtracted from the normalized viable intensity value). The above distribution showed that in the upper portion of the biofilm in particular the upper quartile was found the highest concentration of viable cells, the inverse was apparent in the lower portion of the biofilm where nonviable cells were more abundant.

Figure 4.8e: The depth into a photosensitised (L+S+) *S. sanguinis* biofilm as determined by CLSM versus the normalised image intensity for both the viable and nonviable channels.

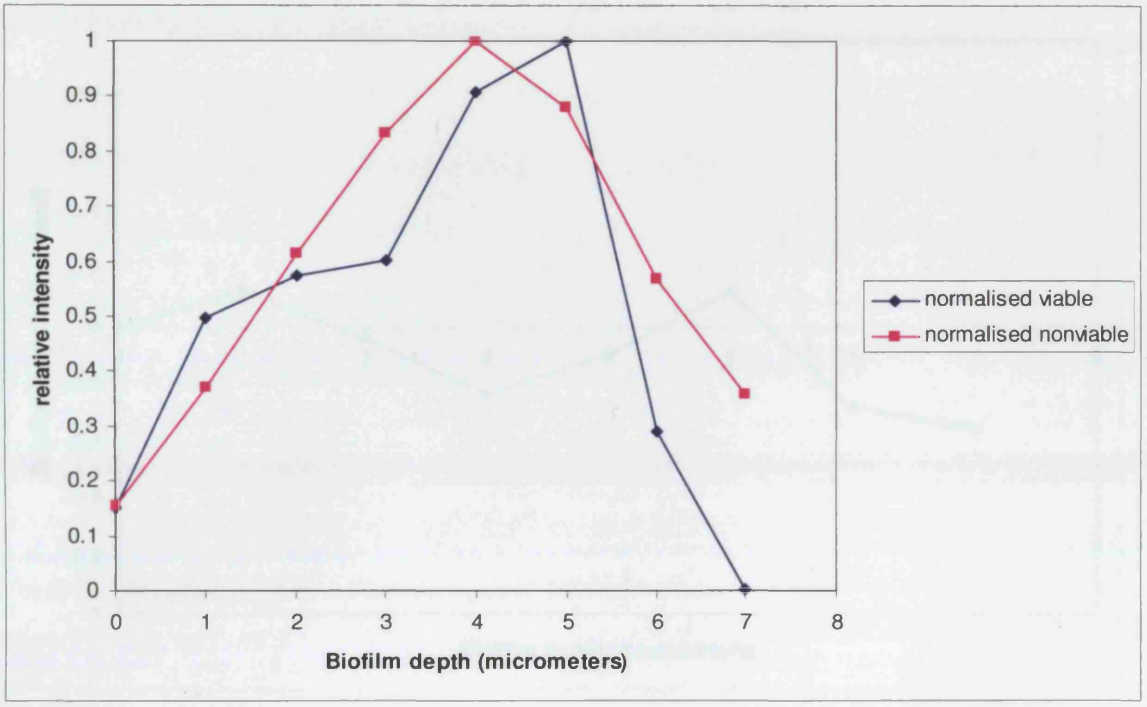


Figure 4.8e shows the relative image intensity for both the viable and nonviable cells of a sensitised biofilm. The image intensity of the photosensitised biofilm did not reveal any apparent overall trend of viable to nonviable cell distribution throughout the z-plane of the biofilm. The peaks of the intensity distribution did not demonstrate the off-set distribution that was evident in the pristine biofilm. Indeed it appeared that subjection to lethal photosensitisation had an equilibrating effect on the distribution of both viable and nonviable cells.

Figure 4.8f: The changes in the viable to nonviable relative image intensity (using normalised data) as a function of biofilm depth in a photosensitised (L+S+) *S. sanguinis* biofilm as determined by CLSM.

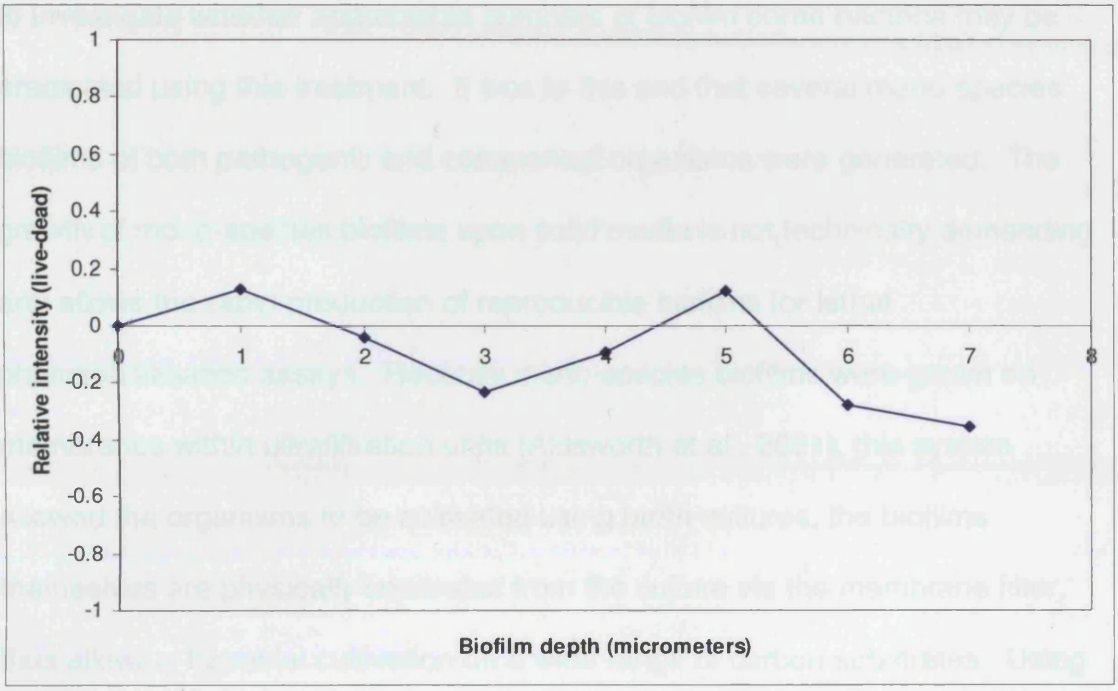


Figure 4.8f shows the relative intensity throughout the photosensitised biofilm. The distinctive distribution that was present in the control biofilm was absent in the biofilm that has been subjected to lethal photosensitisation. The overall ratio of viable to nonviable cells fluctuated upon penetration deeper into the biofilm.

4.4 Discussion

Within the oral cavity, the commensal microflora accrete to form a biofilm (plaque) that harbours a heterogeneous collection of microorganisms, including potentially pathogenic species, implicated in caries and periodontal diseases. In the previous chapter the phenothiazine TBO was found to be a competent

photobactericidal agent of planktonic periodontal pathogens and the primary coloniser *Streptococcus sanguinis.* If photodynamic therapy is to be an efficacious treatment of periodontal diseases then it is of paramount importance to investigate whether appreciable numbers of biofilm borne bacteria may be eradicated using this treatment. It was to this end that several mono-species biofilms of both pathogenic and commensal organisms were generated. The growth of mono-species biofilms upon solid media is not technically demanding and allows the rapid production of reproducible biofilms for lethal photosensitisation assays. Recently mono-species biofilms were grown on membranes within ultrafiltration units (Aldsworth et al., 2001), this system allowed the organisms to be cultivated using broth cultures, the biofilms themselves are physically separated from the culture via the membrane filter, thus allowing bacterial cultivation on a wide range of carbon substrates. Using this culture method, the effect of exogenous nutrient conditions upon a mono-species biofilm could be determined. Moreover, this system could be used to investigate possible cause and effect relationship between the prevailing nutrient conditions and the lethal photosensitisation of mono-species biofilms. Indeed there is a known correlation between nutrient availability and recalcitrance to antimicrobial agents (Brown et al., 1985). Using a similar methodology to the one used in this chapter Dobson et al., 1992 cultivated several oral bacteria as mono-species biofilms to determine the efficacy of a number of photobactericidal agents. The exact numbers of bacteria eradicated was not determined but this study demonstrated that TBO eliminated the test organisms under the

experimental conditions used. Bacterial species examined included the putative periodontopathogens *A. actinomycetemcomitans, F. nucleatum* and *P. gingivalis.* The results recorded in this chapter showed that both pTBO and sTBO killed significant numbers of all the test organisms, this concurred with the findings of Dobson et al., 1992. In another study, biofilms of the commensal organism *Streptococcus intermedius* were cultivated using extracted pre-sterilised teeth (Seal et al., 2002), TBO was used as the photosensitising compound at concentrations of up to 100 μg/ml with a light dose of 21 J. A reduction in recovered viable bacteria of up to 99.999% was observed. However, at this concentration of TBO, significant toxicity in the absence of light was observed, something not evident at the concentrations used in this study.

There was no observable difference in efficacy between the two grades of TBO. Interestingly it appeared cultivating the organisms as rudimentary biofilms had an equilibrating effect upon the photobactericidal action of TBO. When cultivated as planktonic organisms, there was an observable difference in killing between the organisms. This variation was not evident when these organisms formed mono-species biofilms and were subsequently subjected to lethal photosensitisation. When *S. sanguinis* was cultivated as a biofilm, a substantial increase in the light dose administered (~ 15 fold) was necessary to eradicate appreciable numbers of bacteria when compared to the planktonic form. This demonstrated the increased recalcitrance to photosensitisation of bacterial biofilms compared to their planktonic counterparts. Indeed when *F. nucleatum* was grown as a

planktonic organism and subjected to lethal photosensitisation no viable organisms were evident post exposure. Moreover, using ten times the concentration of TBO and increasing the administered light dose c. 15 fold it was still not possible to obtain the result obtained with the planktonic organism, aptly demonstrating the increased recalcitrance of surface associated organisms (Coquet et al., 1998). Under the experimental conditions used in this part of the investigation, for the *S. sanguinis* biofilms, 97% killing was observed for both grades of TBO, for the *P. gingivalis* biofilms, 96.5% kill was recorded using pTBO and 96% kill using sTBO. For the *F .nucleatum* biofilms, 97% kill and 98% kill was recorded for pTBO and sTBO respectively. It appears that these surface associated cells are more resistant to the photobactericidal effect of TBO. However, the kills that were recorded for all test organisms grown as biofilms still represent an appreciable reduction in recovered viable bacteria compared to the control (L-S-).

The CLSM analysis revealed that the *P. gingivalis* biofilms appeared morphologically to be consistent with reports of biofilm architecture (Pratten et al., 2000a). Upon subjection to lethal photosensitisation a qualitative increase in the numbers of dead to live bacteria, when compared to the nascent biofilms, was observable. Analysis of the *S .sanguinis* biofilms using the viability profiling as outlined by Hope et al 2002 showed that in the control biofilm the spatial distribution of cells along the z-plane mimicked the distribution found in microcosm biofilms, where the largest concentration of viable cells was in the

upper portion of the biofilm leaving the lower half of the biofilm with proportionally more nonviable cells. This equilibrium was disturbed upon lethal photosensitisation resulting in no discernible pattern in the distribution of viable to nonviable cells throughout the biofilm. The lethal photosensitisation of the biofilm may result in the eradication of a large number of cells as well as damage to the EPS structure of the biofilm, hence to find the spatial distribution pattern differed between the control and the photosensitised biofilm is unsurprising.

The results of this investigation show that it was possible to cultivate reproducible monospecies biofilms, of both pathogenic and commensal organisms. These biofilms were equally susceptible to the photobactericidal action of both Sigma and pharmaceutical grade TBO in conjunction with laser light. However, to achieve appreciable kill of the biofilm grown organisms, significantly larger light doses were required than was necessary to kill large numbers of the corresponding planktonic organisms. The biofilms generated appeared to be structurally similar to previous biofilms generated *in vitro* and shared a similar viable to nonviable cellular distribution throughout the z-plane of the biofilm. The distinctive spatial distribution of viable and nonviable cells was disrupted on lethal photosensitisation of the biofilms. Qualitatively lethal photosensitisation of the biofilms resulted in higher proportions of nonviable cells compared to the control (L-S-) biofilm.

CHAPTER FIVE

LETHAL PHOTOSENSITISATION OF MEMBRANE GROWN MULTI-SPECIES BIOFILMS

5.1 Introduction

In the previous chapter biofilms composed of a single oral species were cultivated on membrane filters and then subjected to lethal photosensitisation, to determine the effect of both general purpose (Sigma) and pharmaceutical grade TBO on rudimentary mono-species biofilms. In keeping with this incremental approach, multi-species oral biofilms were formed that were derived from human saliva, saliva being a repository for many of the supragingival and subgingival organisms found within the oral cavity. The bacteria which are found in human saliva are there as a result of the removal of organic material and the associated organisms from biofilms, indeed shear forces inherent within the oral cavity due to mastication and tongue movement result in the removal of bound bacteria. The experiments described within this chapter were designed to build on what was determined in chapter four. The biofilms subjected to lethal photosensitisation (again using both sigma and pharmaceutical grade TBO) were multi- and not mono-species. Multi-species biofilms are more representative of the biofilms encountered within the oral cavity (Marsh., 1994), the aims of this section of the research project, therefore, were to investigate whether multi-species biofilms possessed significantly increased resistance to photodynamic action compared to mono-species biofilms.

5.2 Materials and methods

5.2.1 Collection and Preparation of pooled saliva samples

The method used was the same as that described in chapter 2, section 2.1.2.2.

5.2.2 Growth of biofilms on membrane filters

The method used to prepare membrane filters was the same as that described in chapter 2, section 2.4.1.

The method used to cultivate the multi-species biofilms was as follows:

1) A cryotube containing pooled saliva was removed from the -70 °C freezer (Lab Impex Research) and allowed to thaw.

2) The 5 mm diameter cellulose nitrate discs were then added to the petri plates containing FAA (Bioconnections). Each disc was inoculated with 25 μl of the bacterial suspension. The plates were then placed in an anaerobic cabinet.

5.2.3 Bacteriological characterisation of membrane filter cultivated biofilms

The method used was the same as that described in chapter 2, section 2.3.

5.2.4 Preparation of TBO

This was carried out as described in chapter 2, section 2.6.1.1

5.2.5 Susceptibility of membrane grown biofilms to TBO/HeNe laser light

The method used was the same as that described in chapter 2, section 2.6.3.

5.2.6 CLSM of membrane filter biofilms

The method used was the same as that described in chapter 2, section 2.5

5.2.7 Analysis of the CLSM data

The method used was the same as that described in chapter 2, section 2.5.1 with the following exception.

The CLSM image stack data was analyzed by Leica Confocal Software Simulator SP, version 2.00 (Leica Microsystems Heidelberg GmbH, Germany) to produce surface renderings. The surface topography of the scanned biofilms was subject to processing to allow visualisation of the macroscopic features of the biofilm without hinderance of the finer surface details.

5.2.8 Statistical analysis of the data

The statistical significance of the data was ascertained using the two-tail t-test assuming unequal variance.

5.3 Results

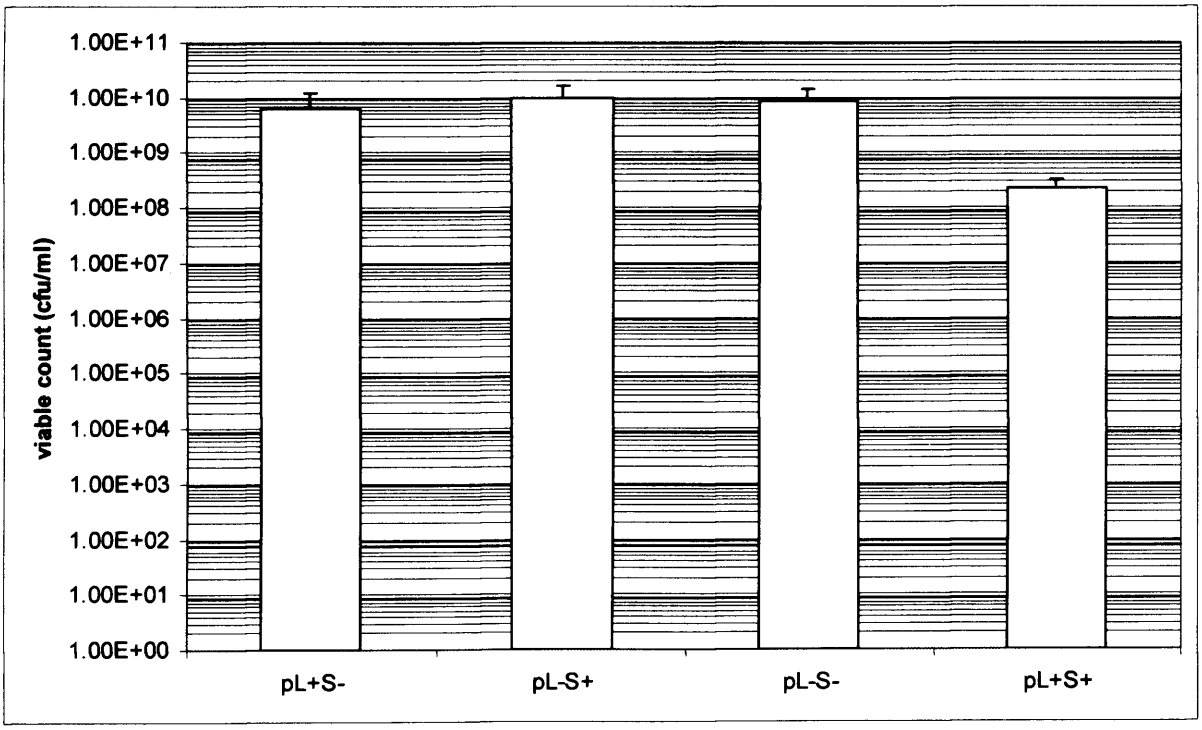
The composition of the membrane grown multi-species biofilms was determined using viable count data on selective and non-selective media and is compiled in table 5.1. Figures 5.1 – 5.4 show viable count data of biofilms subjected to lethal photosensitisation under differing experimental conditions. Confocal microscopy (Figures 5.5 – 5.6) was also performed on biofilms in their native state, and

biofilms which had been photosensitised prior to confocal microscopy being performed.

Table 5.1: The composition of the multi-species biofilms as determined by viable counts using both selective and non-selective agar

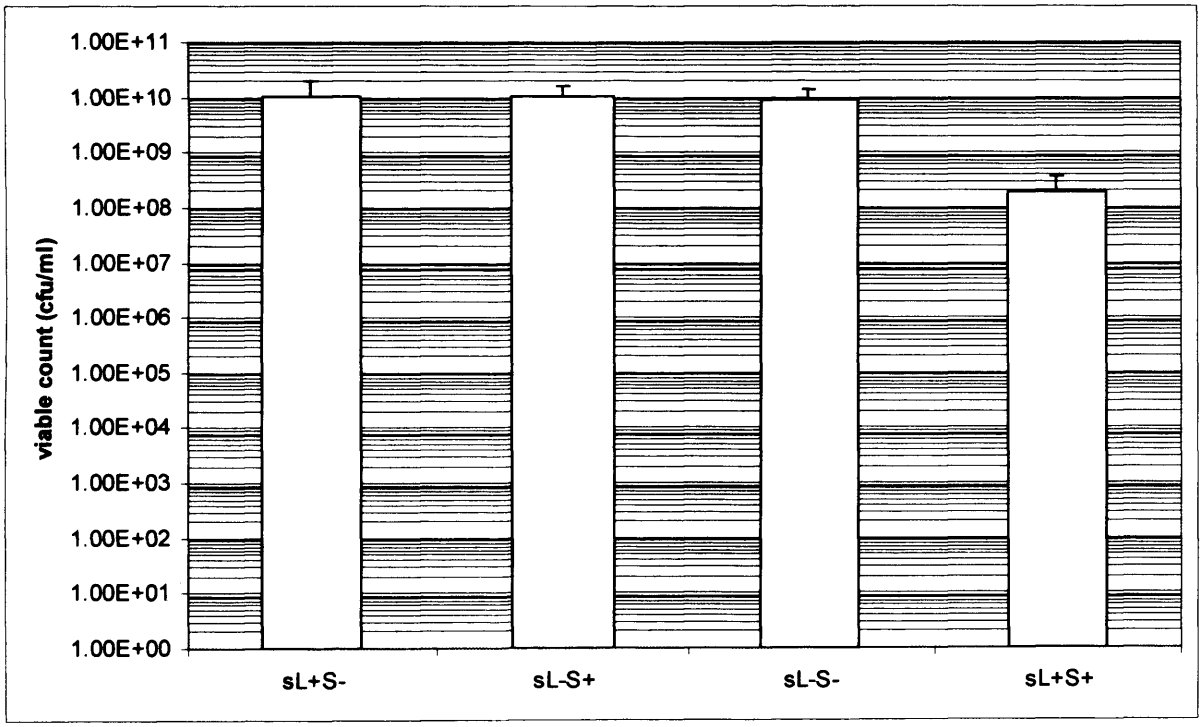
Bacterial Group	% composition of biofilm
streptococci	32.8
Veillonella spp.	6.5
Actinomyces spp.	0.1
Gram-negative anaerobes	0.1

Figure 5.1: Viable counts of multi-species biofilms cultivated on membrane filters exposed to 31.5 J of HeNe laser light (energy density, 81.9 Jcm^{-2}) in the presence of 10 μl of 81.7μM pTBO with appropriate controls.



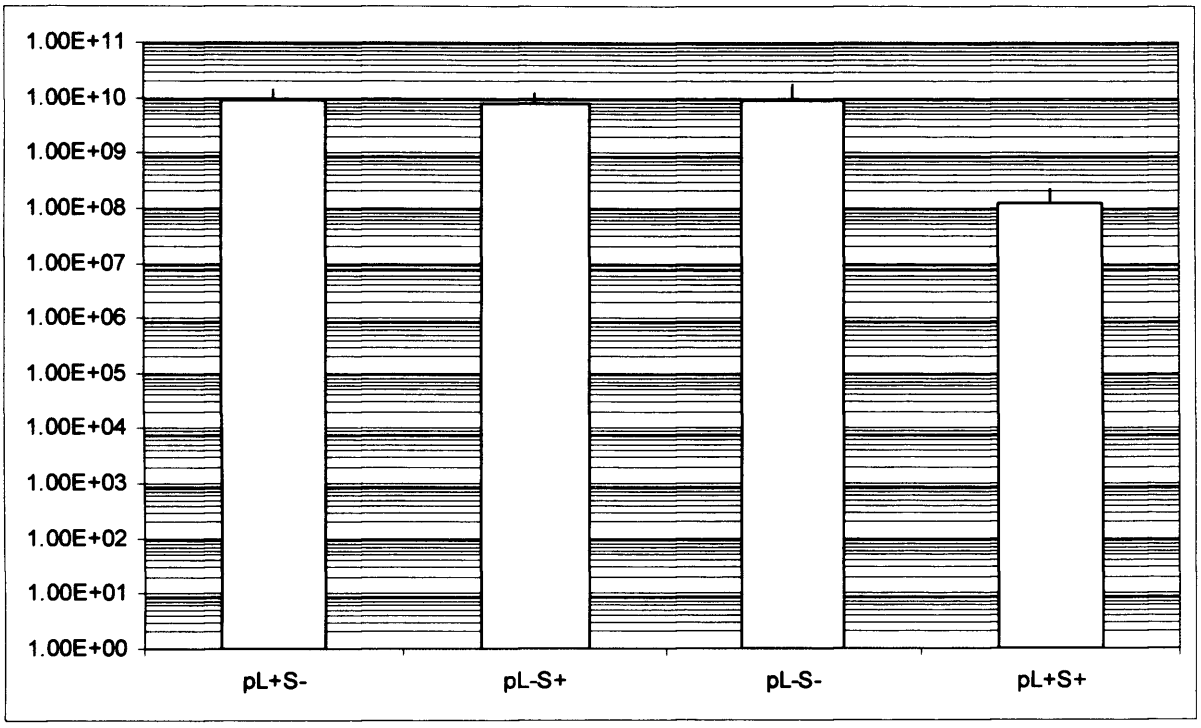
As is seen in figure 5.1 subjection of multi-species biofilms to lethal photosensitisation resulted in a >90% (97.4%) reduction in the number of recovered viable organisms (L+S+) compared to the control biofilm (L-S-). This reduction was found to be statistically significant (P = 0.0002).

Figure 5.2: Viable counts of multi-species biofilms cultivated on membrane filters exposed to 31.5J of HeNe laser light (energy density, 81.9 Jcm^{-2}) in the presence of 10µl of 81.7µM sTBO with appropriate controls.



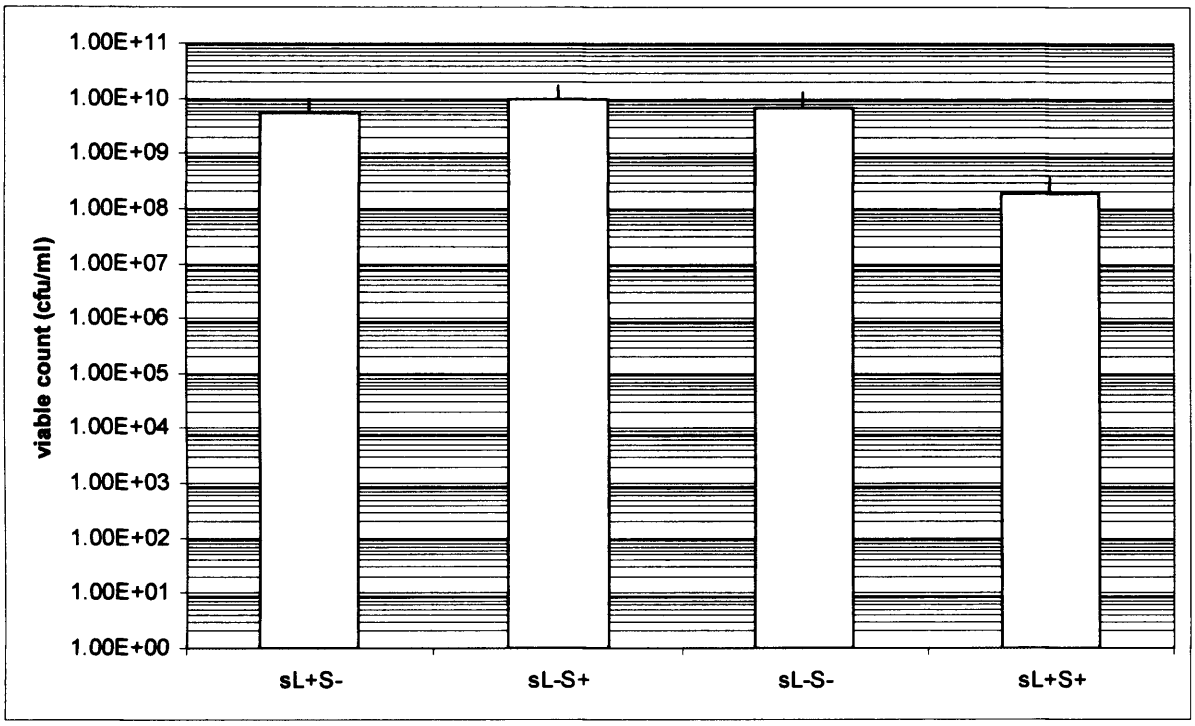
Lethal photosensitisation of multi-species biofilms resulted in a >90% (97.8%) reduction in the number of recovered viable organisms (L+S+) when compared to the control biofilms (L-S-), as may be seen in figure 5.2, indeed this reduction in the number of viable bacteria was found to be statistically significant (P = 0.0000532).

Figure 5.3: Viable counts of multi-species biofilms cultivated on membrane filters exposed to 63 J of HeNe laser light (energy density, 163.7 Jcm^{-2}) in the presence of 10µl 81.7µM pTBO with appropriate controls.



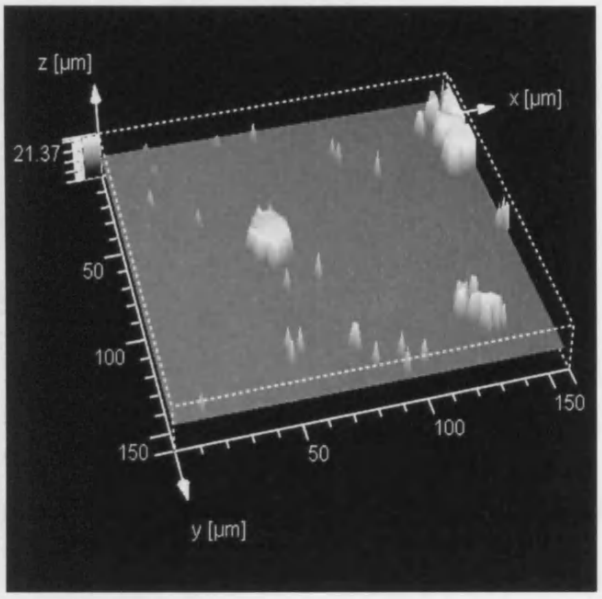
Exposure times were doubled from 15 minutes to 30 minutes to determine whether this led to a concomitant increase in the proportion of bacteria that were killed within the biofilm. As is evident from figure 5.3 whilst lethal photosensitisation led to a statistically significant >90% (98.6%) (P= 0.00183) reduction in the number of recovered viable organisms (L+S+) when compared to the control biofilms (L-S-), the increase in exposure to laser light did not result in a marked decrease in the number of recovered viable bacteria, when compared to the shorter exposure time.

Figure 5.4: Viable counts of multi-species biofilms cultivated on membrane filters exposed to 63 J of HeNe laser light (energy density, 163.7 Jcm^{-2}) in the presence of 10µl 81.7µM sTBO with appropriate controls.



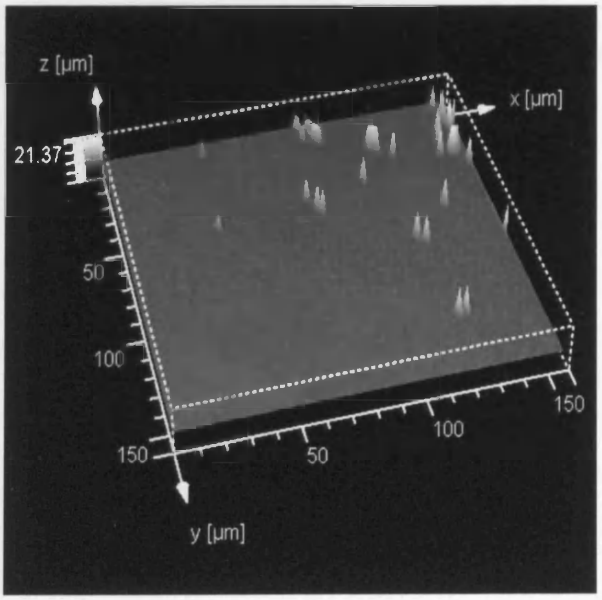
When using sTBO rather then pTBO the same trend was evident as was seen in figure 5.4. Increasing the exposure time from 15 to 30 minutes did not decrease the number of recovered viable bacteria post exposure, the number of organisms killed by lethal photosensitisation was not increased. However lethal photosensitisation of the multi-species biofilms resulted in a statistically significant >90% (97.2%) (P= 0.002) reduction in the number of recovered viable organisms (L+S+) when compared to the control biofilms (L-S-).

Figure 5.5a: CLSM of an untreated biofilm (L-S-) showing the distribution of the live bacteria within the biofilm.



The CLSM data revealed qualitative differences between the distribution and relative abundance of dead to live bacteria in the untreated biofilm. What was visualised in both CLSM images was not the membrane filters, but a virtual surface which was constructed via a levelling effect applied by the computer. Whilst three stack like structures were evident in the live channel (figure 5.5a) these structural motifs were lacking in the dead channel (figure 5.5b), with only one cluster that was seemingly evident in both the live and dead channels. Hence the biofilm stack structures that were evident appeared to be composed of predominantly live bacteria within the untreated biofilm.

Figure 5.5b: CLSM of an untreated biofilm (L-S-) showing the distribution of the dead bacteria within the biofilm.



The CLSM data extrapolated from a biofilm subjected to lethal photosensitisation (L+S+) revealed qualitative differences between the distribution and relative abundance of dead to live bacteria within the biofilm. Lethal photosensitisation appeared to result in the number of dead bacteria (figure 5.6b) increasing relative to the number of live bacteria (figure 5.6a). Hence four stack structures (figure 5.6b labelled 1,2,3, and 4) were evident in the dead channel whereas only one stack was evident in both live and the dead scans (figure 5.6b labelled 4), this stack was composed of large numbers of both live and dead bacteria

Figure 5.6a: CLSM of a treated biofilm (L+S+) showing the distribution of the live bacteria within the biofilm. Biofilms were exposed to 31.5J of HeNe laser light (energy density, 81.9 Jcm^{-2}) in the presence of 10µl of

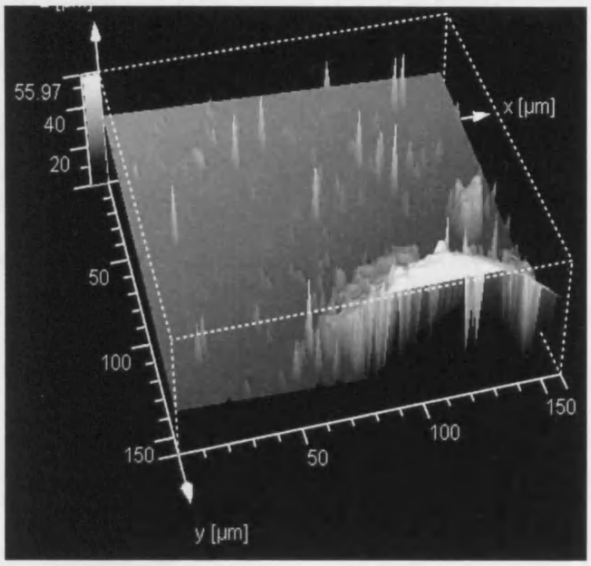


Figure 5.6b: CLSM of a treated biofilm (L+S+) showing the distribution of the dead bacteria within the biofilm. Biofilms were exposed to 31.5J of HeNe laser light (energy density, 81.9 Jcm^{-2}) in the presence of 10µl of 81.7µM pTBO.

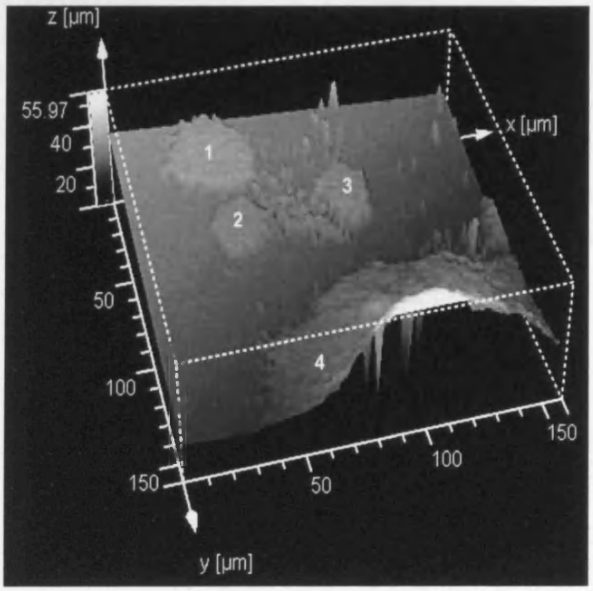


Figure 5.7a: The depth into a control (L-S-) multi-species biofilm as determined by CLSM versus the normalised image intensity for both the viable and nonviable channels.

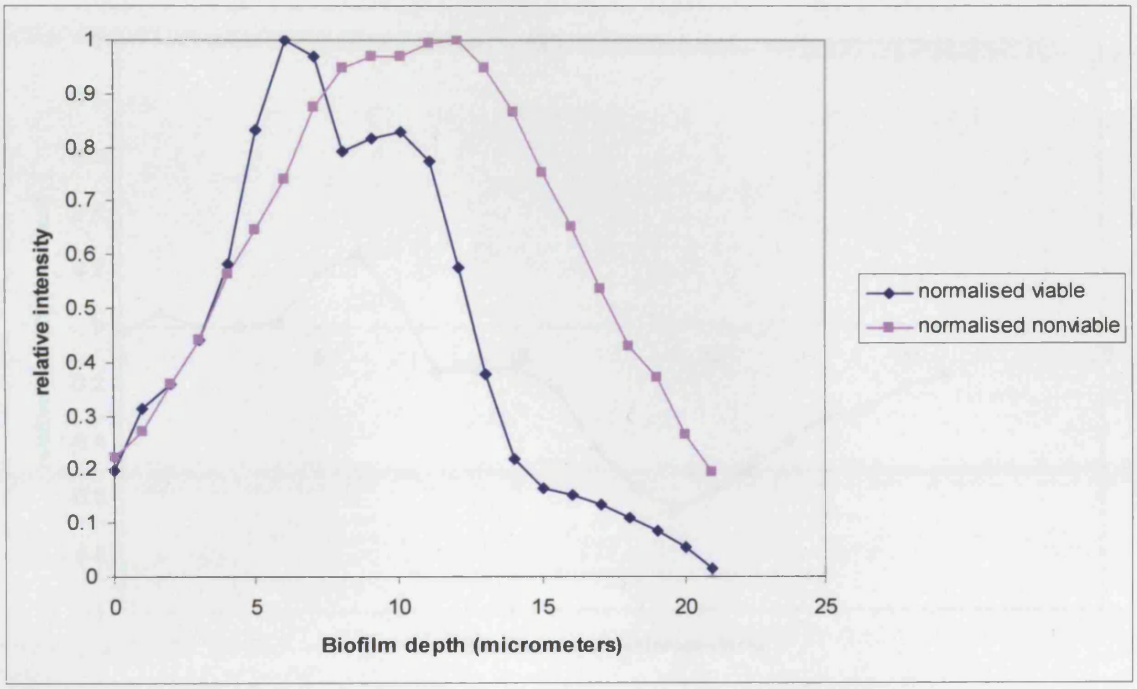


Figure 5.7a shows the normalised image intensity for both the viable (live) and nonviable (dead) channels; whilst the highest intensities of both the viable and nonviable cells seemed to be concentrated within the centre of the biofilm.

Figure 5.7b: The changes in the viable to nonviable relative image intensity (using normalised data) as a function of biofilm depth in a control (L-S-) multi-species biofilm as determined by CLSM.

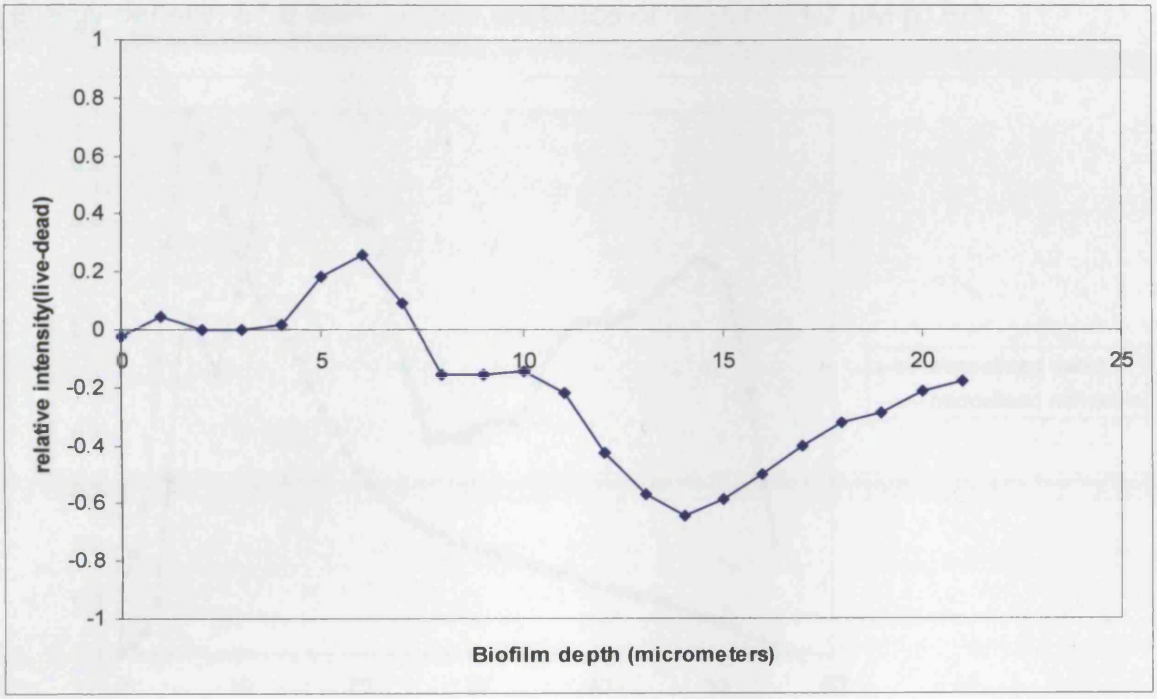
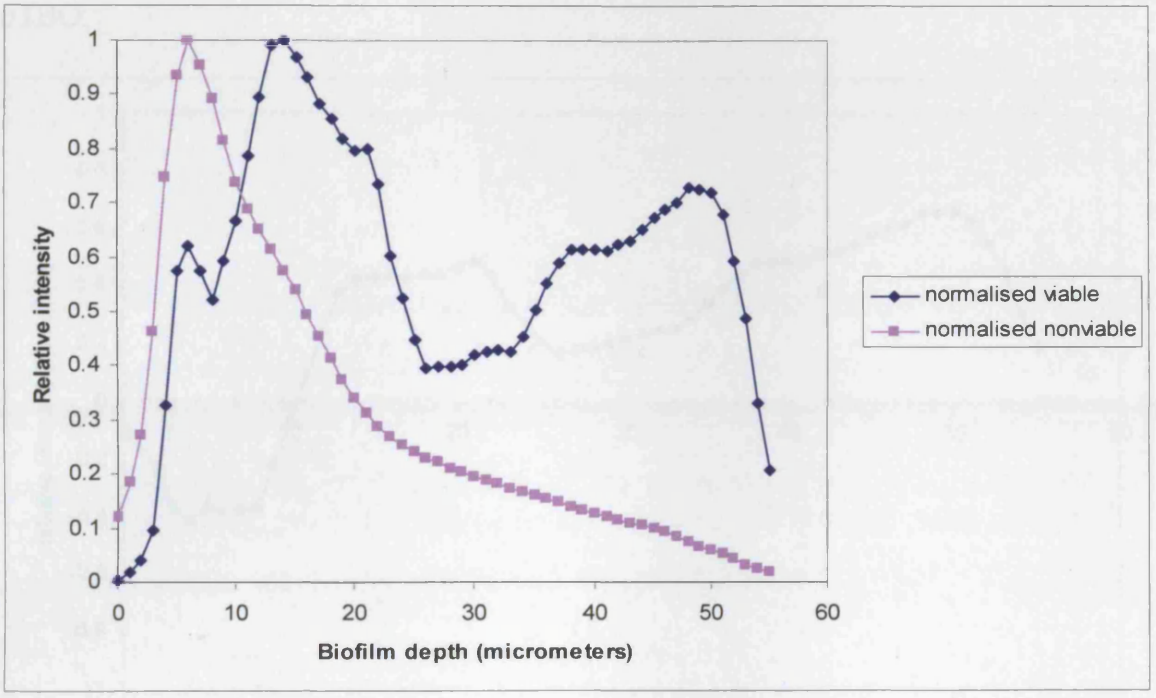


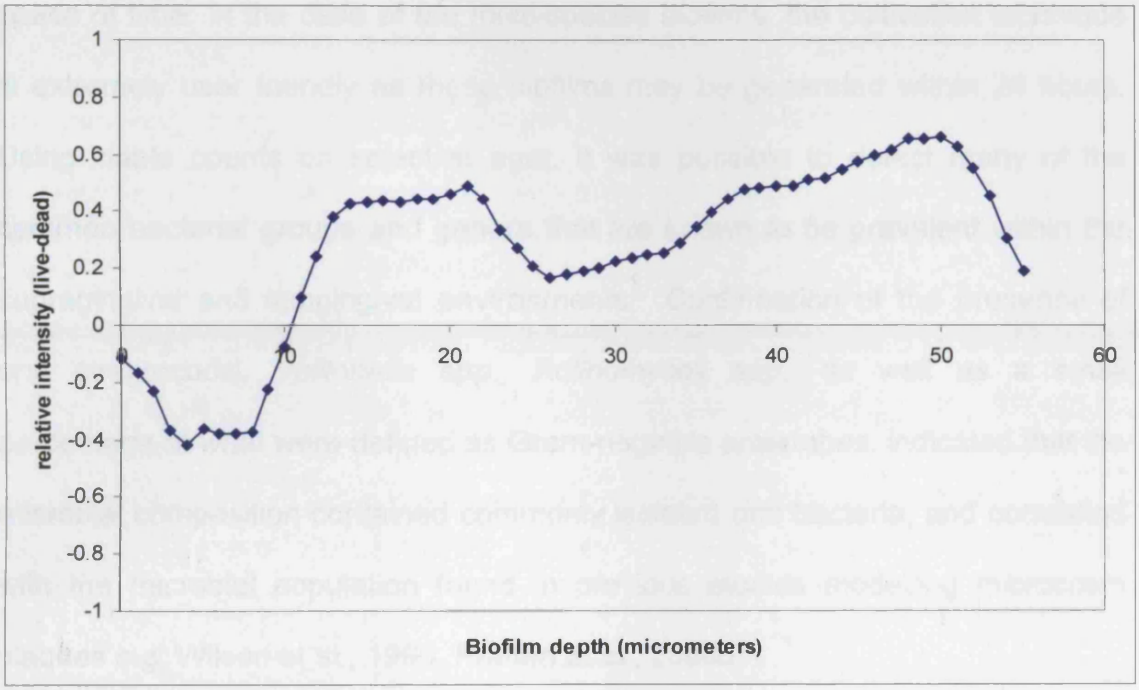
Figure 5.7b shows the relative intensity of the viable to nonviable cells throughout the z plane of the biofilm. Immediately evident was the higher concentration of viable cells in the upper half of the biofilm. This situation was reversed on progression through the z plane of the biofilm as there were proportionally more nonviable cells proximal to the biofilm filter membrane interface.

Figure 5.8a: The depth into a photosensitised (L+S+) multi-species biofilm as determined by CLSM versus the normalised image intensity for both the viable and nonviable channels. Biofilms were exposed to 31.5 J of HeNe laser light (energy density, 81.9 Jcm^{-2}) in the presence of 10 µl of 81.7 µM pTBO.



In figure 5.8a we see the normalised image intensity of both the viable and nonviable channels throughout the z plane of the biofilm. The biofilm was subjected to lethal photosensitisation, the highest concentration of both viable and nonviable cells was in the upper half of the biofilm. Interestingly in a reverse of what was evident in the control biofilm the highest normalised image intensity in the nonviable channel occurred closer to the biofilm filter membrane interface than does the highest normalised image intensity in the viable channel.

Figure 5.8b: The changes in the viable to nonviable relative image intensity (using normalised data) as a function of biofilm depth in a photosensitised (L+S+) multi-species biofilm as determined by CLSM. Biofilms were exposed to 31.5 J of HeNe laser light (energy density, 81.9 Jcm^{-2}) in the presence of 10 µl of 81.7 µM pTBO.



In figure 5.8b is seen the relative image intensity of viable to nonviable cells throughout the z plane of the biofilm. Here it was apparent that in the uppermost portions of the photosensitised biofilm was found the highest concentration of nonviable cells, as mentioned previously this marked a reversal of what was found with the untreated multi-species biofilms

5.4 Discussion

Using the same rationale as employed in chapter four, but substituting pooled human saliva for mono-species organisms, it proved possible to cultivate multi-species biofilms using the membrane filter technique. As pointed out previously, this approach is desirable as it allows the biofilm generation in a relatively short space of time. In the case of the multi-species biofilms, the cultivation technique is extremely user friendly as these biofilms may be generated within 24 hours. Using viable counts on selective agar, it was possible to detect many of the common bacterial groups and genera that are known to be prevalent within the supragingival and subgingival environments. Confirmation of the presence of oral streptococci, *Veillonella* spp., *Actinomyces* spp., as well as a small percentage of what were defined as Gram-negative anaerobes, indicated that the microbial composition contained commonly isolated oral bacteria, and correlated with the microbial population found in previous studies modelling microcosm plaques e.g. Wilson et al., 1998, Pratten et al., 2000b.

It should be noted that there were minor problems associated with cultivating biofilms on filter membranes. Frequently on seeding the membrane filters excess inoculum rolled off the filter membrane and onto the medium, hence the biomass was not restricted to the filter itself and when the filter was removed from the agar surface the additional biomass would often, but not always, remain attached to the filter membrane. This may have caused fluctuations in the number of organisms present on, or attached to, a particular membrane filter.

The filter membranes themselves, post-autoclaving, sometimes became warped even though the membranes were immersed in PBS to try and prevent this. Upon biofilm cultivation, the biofilm assumed the shape of the membrane. Hence, when photosensitiser was added, uniform distribution of the photosensitiser on the biofilm was problematic.

Upon subjection of multi-species biofilms to lethal photosensitisation, appreciable kills were evident. Consistently the kills obtained were of the order of between 1 $\log_{10}$ and 2 $\log_{10}$ (constituting a 90-99% reduction in the number of recovered viable bacteria). No observable difference in efficacy was evident when using pTBO or sTBO, in terms of microbial kill. Perhaps if there were any minute differences in efficacy, the use of viable counts did not provide sufficient resolution for these to be apparent. Doubling the light dose administered to the biofilms, from 31.5 J to 63 J did not result in an increase in kill. This was surprising as it was anticipated that increasing the light dose would have caused a concomitant increase in the bactericidal effect. Indeed, doubling of the administered light dose produced a percentage kill that was almost identical in most instances to the kill obtained using the lower light dose. The increased light dose was delivered using a longer exposure time without alteration of the power density of the laser. Possibly the use of a higher power density delivering the same light dose (63 J) but over a shorter period of time might have resulted in an increase in the observable kill (Wainwright, 1998), or possibly excess TBO on the surface may have hindered light penetration so no increased kills were observed.

What was evident from the results of both chapter four, and this chapter, is the relative recalcitrance demonstrated by the filter membrane biofilms to photodynamic therapy when compared to planktonic oral organisms. When planktonic oral organisms were subjected to lethal photosensitisation, a difference in kill was very evident depending on the organism being photosensitised. This was due to factors such as the nature of the cell wall of the organism. When these same organisms were present as biofilms, the kill achieved became independent of the organism composing the biofilm. In this chapter, multi-species biofilms have been shown to be as recalcitrant to the photodynamic action of TBO as mono-species biofilms, the results obtained being very similar. Hence, it appeared that the composition of the biofilm (at least those comprised of oral species) does not have a bearing on its susceptibility to photodynamic therapy, recalcitrance to photodynamic therapy being independent of biofilm composition. One may have envisioned that as the biofilms became more diverse, their resistance to PDT would have increased, due to the constituent bacteria having differing susceptibilities to the photodynamic action of TBO. However, it appears that the mere fact that the organisms were cultivated as a biofilm resulted in increased resistance to lethal photosensitisation. It is well established that biofilms exhibit decreased susceptibility to many antimicrobial agents, and it appears that biofilms also have decreased susceptibility to the photodynamic action of TBO and HeNe laser light, when compared to planktonic organisms. However, it should be noted that

appreciable numbers of biofilm organisms may still be killed using photodynamic therapy.

Studies using CLSM provided some very interesting data on the effect of PDT upon multi-species biofilms, the viability profiling coupled with the qualitative biofilm topography data generated from the biofilms, permitted the visualisation of the effect of photosensitisation. The photosensitised biofilms (L+S+) possessed larger quantities of nonviable cells compared to viable cells. When the control biofilm (L-S-) was analysed the reverse situation was apparent, qualitatively, there were more biofilm 'stacks' with appreciable live signal than dead. When the viability profiling was analysed, an interesting trend was revealed. Within the control biofilm, it was apparent that there were larger numbers of viable bacteria in the upper half of the biofilm, compared to the lower half, which contained a higher proportion of nonviable bacteria. This concurred with the findings of Hope et al 2002, who reported this same trend for pristine microcosm biofilms. However, when the photosensitised biofilm was analysed the viability profile was drastically different. Larger numbers of dead bacteria in the upper portion of the biofilm were found, especially in the area that is proximal to the biofilm surface interface. However, on progression to the base of the biofilm, the opposite occurred with proportionally more viable than nonviable organisms. Thus, photosensitisation only seemed to occur predominantly in the upper portion of the biofilm, whereas in the lower potion, the organisms seemed to be impervious to photosensitisation. It is possible that the cells in the upper

portion of the biofilm formed a protective screen, protecting the cells deeper within the biofilm; thus, the organisms proximal to the surface may have been rapidly photosensitised but then served to prevent penetration of the laser light deeper into the biofilm. Indeed this 'protective screen' may have also served to inhibit the diffusion of the photosensitiser, hindering access to the centre of the biofilm and thus preventing photosensitisation. This is one possible explanation that may account for the increased recalcitrance of biofilms to photosensitisation.

The results of this investigation have shown that it is possible to cultivate both representative and reproducible multi-species biofilms using the membrane filter technique. These biofilms were susceptible to photosensitisation using TBO and HeNe laser light. No difference in efficacy was evident between sTBO and pTBO. Increasing the administered light dose did not lead to a concomitant increase in kill. The viability profile of the control biofilm was similar to that previously described for other microcosm biofilms grown *in vitro.* Upon photosensitisation of the biofilms, the viability profile was inverted demonstrating that photosensitisation occurs in the uppermost portions of the biofilm.

In the chapters to follow an *in vitro* model, the CDFF, was employed to generate biofilms derived from subgingival plaque, the CDFF was suited to this task as specific growth parameters may be controlled, this model also allowed the simultaneous generation of up to 75 biofilms per run. The membrane filter method of cultivating biofilms was rapid, however it did not permit the large-scale

generation of steady-state biofilms under defined and controllable growth conditions.

CHAPTER SIX

COMMUNITY ANALYSIS OF CDFF CULTIVATED SUB-GINGIVAL BIOFILMS

6.1 Introduction

At the outset of this investigation the initial objective was to identify a suitable photobactericidal agent from a variety of candidates, TBO appeared preferable to the others tested as it proved capable of killing appreciable numbers of planktonic oral organisms, these organisms were then cultivated as mono-species biofilms, using membrane filters, these biofilms proved susceptible to photosensitisation. Multi-species biofilms were formed, using membrane filters, these also proved susceptible to lethal photosensitisation using TBO and HeNe laser light. However, in order to fully evaluate the likely efficacy of PDT in treating periodontal diseases *in vivo* it was necessary to develop a laboratory model that was capable of producing large numbers of biofilms that mimicked (in terms of composition and structure) the subgingival plaques found in periodontitis patients *in vivo.* Using the filter membrane method it was not possible to easily generate a large number of steady-state biofilms under defined and controllable growth conditions. The chosen model, the CDFF (see section 1.4.1 and 2.2), facilitated the cultivation of mixed microcosm communities, and may be set-up to closely approximate both the biotic and abiotic conditions *in-situ* within a periodontal pocket.

Upon generation of the mixed microbial communities, derived from periodontal plaque, several analyses were performed on the biofilm community. The study of microbial communities has previously centred upon cultivation and identification. The available techniques were time-consuming and, crucially, only permitted

analysis of the cultivable microbiota (Spratt et al., 1999). Techniques available allow the circumvention of such problems, permitting the examination of both the genetic and functional characteristics of the entire microbial community. One such technique is Community Level Physiological Profiling (CLPP) this is based upon substrate utilisation of the microbial community. The CLPP analyses are performed using BIOLOG plates, these were originally developed to allow identification of mono-species bacterial cultures, but were subsequently employed to produce a biochemical fingerprint of an entire microbial community (Garland et al., 1991) and have subsequently been extensively used to study microbial communities, in environments as diverse as the potato phyllosphere (Heuer et al., 1997) and contaminated sawmill soil (Laine et al., 1997). CLPP analysis is based upon the ability of the microbial community to metabolise 95 different carbon sources (each of which may be considered a discrete biochemical test), which may be divided into 10 broad generic groups, polymers, sugars and sugar derivatives, methyl esters, carboxylic acids, amides, amino acids and peptides, nucleosides, amines, alcohols and sugar phosphates. The assay itself yields a pattern dependent upon whether a substrate has been used by the community, hence for a given microbial population, CLPP permits the generation of a (novel) metabolic fingerprint. Metabolic profiling has been employed previously by microbial ecologists. In a study carried out by Campbell et al., (1997), the standard CLPP compounds were supplemented with carbon compounds associated with plant root exudates, facilitating the categorisation of soil microbial communities within the plant rhizosphere. CLPP has previously

been shown to be adept at the characterisation of unknown single species, when utilised to speciate rhizobia, CLPP displaying congruence when compared to genotypic fingerprinting of the organisms (Mc Inroy et al., 1999). Additionally CLPP has been employed to allow comparative analysis between adjacent microbial populations in soil, each occupying a different ecological niche (Lawlor et al., 2000). Finally and most pertinently as regards this study, CLPP has been used to compare the metabolic activity occurring within dental plaque microcosms (Anderson et al., 2002).

Another profiling technique is Denaturing Gradient Gel Electrophoresis (DGGE), which again allows the generation of a fingerprint or profile of complex microbial communities (Muyzer et al., 1993). DGGE provides for separation of partially amplified 16S rRNA gene fragments of the same length but differing sequence (Muyzer et al., 1998). The specific denaturing gradient incorporated into each polyacrylamide gel results in differing migratory distances for each band, the migratory distance travelled is dependent primarily upon the relative guanine and cytosine abundance within the amplified sequence, a GC clamp prevents the complete denaturation of the sequence, which may otherwise result in the formation of single stranded DNA. DGGE has been employed to investigate spatial homogeneity within soil sediment (Boon et al., 2000) and is particularly suited to the monitoring of successional events occurring within microbial communities due to the rapidity with which a profile may be generated. Hence DGGE has been used to determine the impact of biostimulation upon microbial

community structure (Ogino et al., 2001), to analyse community structure and successional events occurring within populations of sulphate-reducing bacteria (Okabe et al., 2002) and to resolve both the structural and functional dynamics within a maturing bacterial biofilm (Santegoeds et al., 1998). Engelen et al., 1998 used a combination of CLPP and TGGE (where the denaturant is replaced by a temperature gradient) to monitor the effect of pesticide treatment upon bacterial soil communities and found both techniques to be at least as good as some of the more traditional assays, complementing what may be garnered via some of the traditional techniques. The methods outlined are of relevance in the study of biofilm formation, both CLPP and DGGE were employed to investigate community development within the CDFF. A thorough analysis of the biofilms produced by the CDFF was thought to be highly desirable before using the model to produce biofilms for subsequent lethal photosensitisation assays.

6.2 Materials and Methods

6.2.1 Collection and Preparation of subgingival plaque samples

This was carried out as described in chapter 2, section 2.1.2.1.

6.2.2 Assembly of the CDFF

The CDFF was assembled as detailed in chapter 2, section 2.2

6.2.3 Preparation of the Growth Medium

The medium consisted of 40% horse serum (not heat-inactivated) in RPMI medium (with L-glutamine), with 5 µg/ml hemin and 0.5 µg/ml menadione. This medium was not autoclavable due to its high protein content. The individual sterile constituents were combined in a level 2 sterile tissue cabinet.

6.2.4 CDFF innocultion

This was carried out as described in chapter 2 section 2.2.4

6.2.5 Gaseous conditions within the fermentor

As described in chapter 2 section 2.2.5

6.2.6 Growth of multispecies biofilms using the CDFF

This was carried out as described in chapter 2 section 2.2.6

6.2.7 Sampling of biofilms from the CDFF

This was carried out as described in chapter 2 section 2.2.7

The CDFF was sampled at the following time intervals post-inoculation; 24 h, 72 h, 168 h, 240 h and 336 h.

The CDFF was set-up and run in an identical manner on two separate occasions allowing the collection of two sets of results, permitting a full comparative analysis of biofilm development.

6.2.8 Community Level Physiological Profiling (CLPP)

Substrate utilisation analysis was performed with Biolog Gram-negative microplates (Biolog Inc. Hayward, CA, U.S.A) containing 95 substrates and the colour redox indicator dye, tetrazolium violet. At each time point, 2 pans containing 10 discs were placed in 30 ml of phosphate buffered saline (PBS) (Oxoid, Ltd) and vortex mixed for a period of 2 min which allowed for full biofilm removal from the discs. Once the organisms had been fully suspended in the PBS, 150 µl was inoculated into each well of duplicate microplates. These were then incubated at 37 ℃, under either aerobic or anaerobic conditions. The colour development within the wells was monitored at an OD of 590 nm (MRX$_{TC}$, Dynex Technologies, Chantilly, Virginia, U.S.A) against a control well, which contained no carbon substrate only the tetrazolium dye. Optical densities were measured for each microplate at 6 h and 24 h time points, post inoculation. A well was regarded as being positive when it yielded a net OD_{590} greater then 140 % of the control (Verschuere et al., 1997)

6.2.9 Denaturing Gradient Gel Electrophoresis (DGGE)

At each sampling point, two discs were removed and frozen at -70 ℃ (Lab Impex Research) to allow DGGE analysis to be performed at a later date. Ten biofilm development time points were sampled in both runs, hence 20 discs were collected in total. DGGE requires DNA extraction followed by two rounds of DNA amplification, the second round of amplification being nested. Integral to the success of DGGE is an appropriate denaturant gradient, to enable band

separation. Therefore a preliminary 'perpendicular' gel was run allowing the determination of the required denaturing gradient for optimal band resolution. Once a suitable denaturant gradient was established a second 'parallel' gel was run incorporating the determined optimal denaturant concentrations. On running the DGGE gel, a banding pattern was evident when stained, each separate band representing a distinct phylotype.

6.2.9.1 Whole genomic DNA extraction

The whole genomic DNA extraction was performed using a commercially available kit (Gentra systems, Minneapolis, U.S.A). Following manufacturers' instructions, briefly, the protocol used for each disc was as follows:

1. 1 ml aliquot of PBS was added to a 1.5 ml tube containing a disc and associated biofilm, this was then vortexed for 2 min to remove the biofilm cells from the disc. The disc was then removed using sterile forceps and the tube centrifuged (Eppendorf U.K) at 8,000 g for 3 min to pellet all of the cells.
2. The supernatant was discarded and 600 µl of cell suspension solution was added to the pellet. The pellet was then re-suspended in the solution.
3. A 3 µl aliquot of lytic enzyme was added and the tubes were then inverted 25 times (to ensure thorough mixing) to equilibrate the enzyme. The tube was then incubated at 37 ℃ for 30 min.
4. The tube was centrifuged for 1 min at 13,600 g.
5. The supernatant was discarded and the pellet re-suspended in 600 µl of cell lysis solution. This was then heated to 80 ℃, for 5 min.

6. A 3 µl aliquot of RNAse was then added, the tube was then inverted 25 times.
7. The tube was then incubated at 37 ℃ for 15 min, and then left to cool at room temperature.
8. A 200 µl aliquot of protein precipitation solution was then added, the tube was then vortex mixed for 20 s, and incubated on ice for 5 min, vortexed for 20 s and left on ice again for 5 min.
9. The tube was then centrifuged at 13,600 g for 3 min, the supernatant was then transferred to another 1.5 ml tube containing isopropanol, the tube was then inverted 50 times.
10. The tube was then centrifuged for 60 s at 13,600 g, the supernatant was then discarded.
11. Six hundred microlitres of 70 % ethanol was added to the pellet, the tube was then inverted 25 times and subsequently centrifuged at 13,600 g for 60 s.
12. The tube was inspected to ensure a pellet was present, the supernatant was discarded and 50 µl of DNA hydration solution was added.
13. The extractions were left overnight to allow the DNA to re-hydrate.
14. Upon DNA hydration the extractions were vortexed, the duplicate extractions were pooled for each time point reducing the number of samples to 10, one per time point for 2 runs.

6.2.9.2 First round PCR amplification

PCR amplification was performed with a Primus 25 Thermocycler (MWG Biotech, Milton Keynes, U.K) using 10x NH_4-based reaction buffer (Bioline, London, U.K) containing 10 mM $MgCl_2$, 400 µM of deoxynucleotide triphosphates, 0.4 pmol of each primer, 5 U *Taq* DNA polymerase (Bioline U.K) and 1 µl of template DNA, this was in a total volume of 100 µl. The 16S rRNA gene conserved primers (see table 6.1) used for this first round PCR were 27F and 1492R (Genosys Biotechnologies Ltd, Cambridgeshire, U.K). The samples were then amplified as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 59 °C for 1 min and 72 °C for 1.5 min, with a final extension at 72 °C for 5 min. The presence of product was confirmed on a 1 % agarose gel (Amresco, NBS Biologicals, Huntingdon, U.K).

Table 6.1: Primer nomenclature and sequences used for the DGGE experiments

Primer	Sequence	Reference
27F[a]	5' – AGAGTTTGATCMTGGCTCAG – 3'	Lane.,1991
1492R[a]	5' – TACGGYTACCTTGTTACGACTT – 3'	Lane.,1991
338F	5' – ACTCCTACGGGAGGCAGCAG – 3'	Lane.,1991
518R	5' – ATTACCGCGGCTGCTGG – 3'	Muyzer et al.1993
GC clamp[b]	5' – CGCCCGCCGCGCGCGGCGGG	
	CGGGGCGGGGGCACGGGGGG – 3'	Muyzer at al.,1993

[a] redundancies are as follows; M= C or A, Y= C or T.

[b]The GC clamp was attached to the 5' end of the 518R primer.

6.2.9.3 Second nested round of PCR amplification

The PCR amplification was performed as described in 6.2.9.2 the template was a 1 in 100 dilution of the first round product. The 16S rRNA gene conserved primers (see table 6.1) used for this nested PCR were 338F and 518R (Genosys Biotechnologies). The 518R primer had a GC clamp attached to its 5' end (see table 6.1). The samples were then amplified as follows: 94 °C for 5 min, followed by 25 cycles of 94 °C for 1 min, 75 °C for 30 seconds and 72 °C for 1.5 min, with a final extension at 72 °C for 5 min. Again the presence for product was verified on a 1 % agarose gel. The PCR products were then purified using a commercial DNA purification kit (Qiagen, Crawley, U.K), producing 30 µl of purified product per sample.

6.2.9.4 DGGE analysis

The DGGE analysis was performed using the Bio-rad DeCode gene system (Bio-rad, Hemel Hempstead, U.K) and is based upon the protocol described by Muyzer et al., 1993. A preliminary perpendicular 0-100 % denaturant gel (100 % denaturant contains 7 M urea and 40 % formamide) was run, to establish optimal denaturant concentration for band resolution, it was established that a gel with a 40-80 % denaturant gradient would result in optimal band separation. The purified PCR products obtained from the second round of PCR were loaded onto 10% (w/v) polyacrylamide gels (suitable for resolving products 200 – 400 bp in

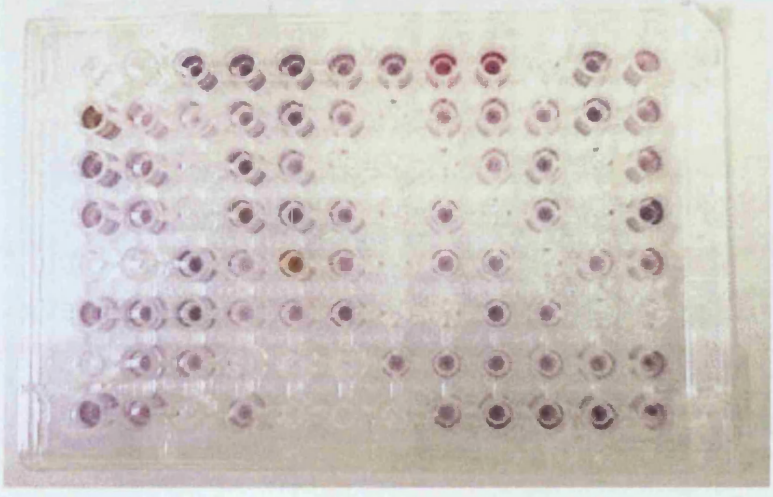
length) in 1 X TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4). The polyacrylamide gels (30 % acrylamide / bisacrylamide gel stock solution 37.5:1, Sigma Ltd) were prepared with a linear denaturing gradient ranging from 40 to 80 %. The electrophoresis was run for 23 hours at 60 °C and a constant 35 volts. Upon completion of the electrophoretic separation, the polyacrylamide gels were immersed for 1 hour in SYBR Green I nucleic acid stain (1:10 000 dilution, Molecular Probes, Eugene, Oregeon, USA). The DNA was visualised using an UV transilluminator operating at 320 nm. Gel images were produced using alphaImager equipment and associated computer software (Innotech Corporation, distributed through Flowgen, Ashby de la Zouch, U.K).

6.3 Results

6.3.1 Results of CLPP analysis

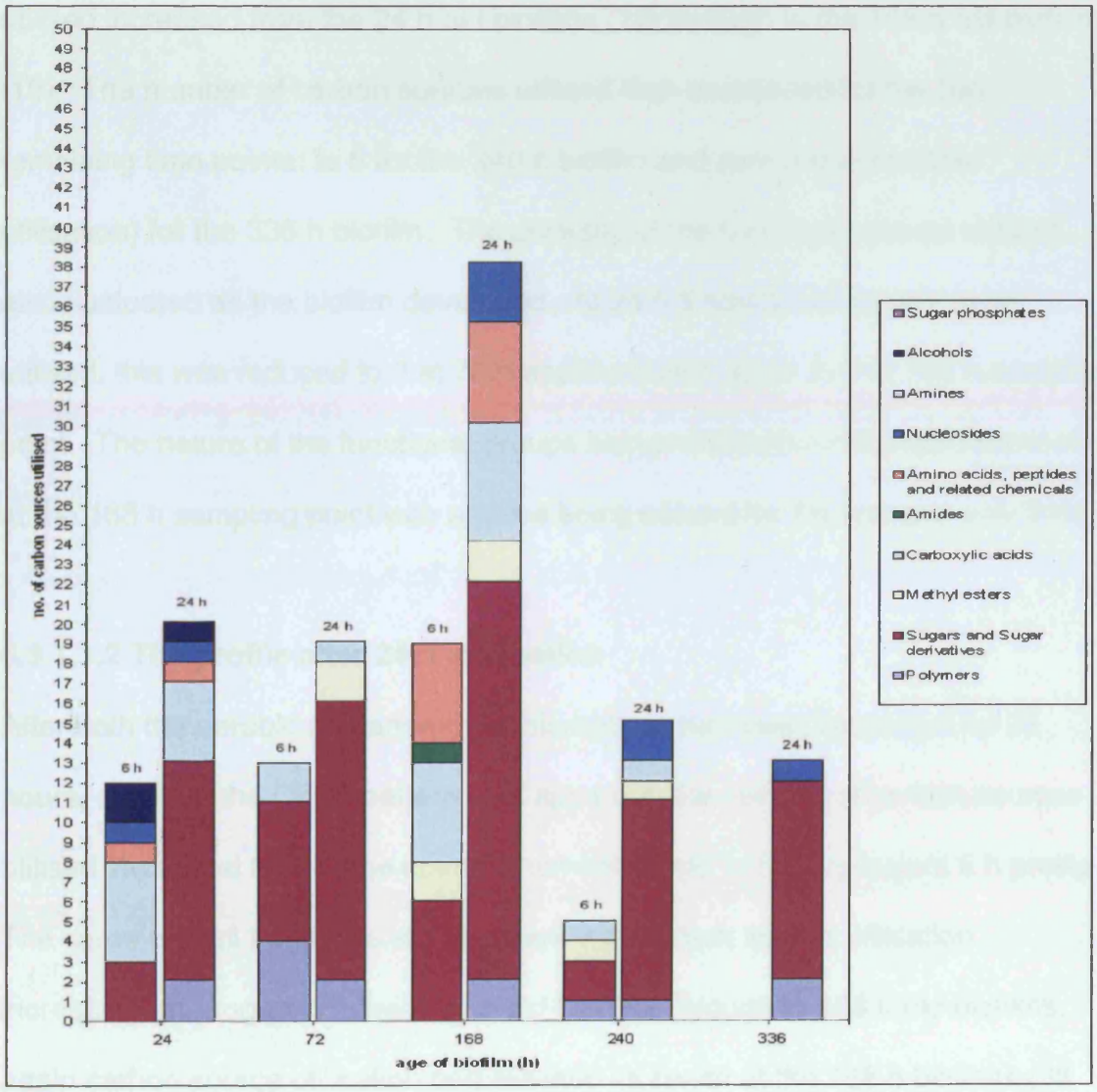
The results of the CLPP analysis for both aerobic and anaerobically incubated plates were combined, but the number of positive wells after 6 h incubation and 24 h of incubation have been shown separately upon a different chart for each run. The bars have been colour coded to represent the differing substrate types and hence the contribution of each functional carbon group to the profile may be seen. In addition a table has been incorporated, detailing the total optical density at each time point for both the aerobic and anaerobically incubated plates, this figure was then adjusted to take into account cell density at each sampling point, producing a normalised figure.

Image 6.1: A photograph showing a Biolog Gram-negative microplate, having being incubated aerobically; as can be seen, some of the wells have developed the distinctive purple colouration indicative of a positive result.



6.3.1.1 Run 1

Figure 6.1: A bar chart showing the number of positive wells for both aerobic and anaerobic plates inoculated with biofilms from run 1, both 6 h and 24 h post-inoculation



6.3.1.1.1 The profile after 6 h incubation

Figure 6.1 shows the number of positive wells 6 and 24 hours post-inoculation for both aerobic and anaerobic microplates. When examining the profile after 6 hours of incubation a trend was identifiable, the number of carbon sources utilised increased from the 24 h old biofilms (12) through to the 168 h old biofilms (19). The number of carbon sources utilised then decreased for the two remaining time points; to 5 for the 240 h biofilm and zero (no detectable utilisation) for the 336 h biofilm. The diversity of the functional groups utilised also fluctuated as the biofilm developed. At 24 h 5 functional groups were utilised, this was reduced to 3 at 72 h and increased again to 5 at 168 h sampling point. The nature of the functional groups being metabolised changed somewhat at the 168 h sampling point with amides being utilised for the first and only time.

6.3.1.1.2 The profile after 24 h incubation

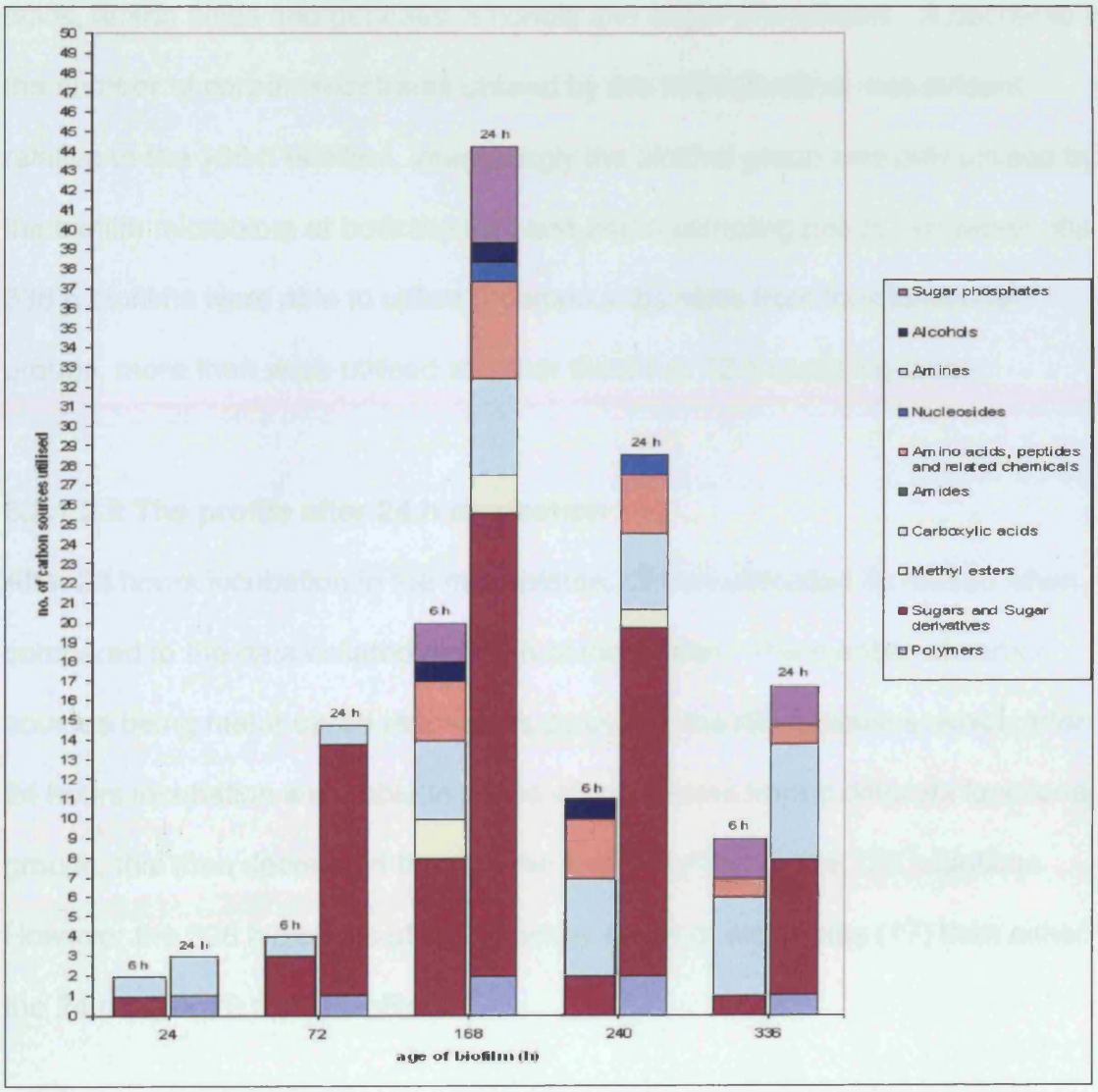
After both the aerobic and anaerobic microplates had been incubated for 24 hours, a shift in the CLPP pattern was apparent, the number of carbon sources utilised increased for all time points when compared to the equivalent 6 h profile. The same overall trend was still apparent with carbon source utilisation increasing on progression from 24 h old biofilms through to 168 h old biofilms, again carbon source utilisation had reached its zenith at the 168 h biofilms (38 substrates utilised) and declined on progression from 168 h to 240 h to 336 h biofilms. What was apparent was the contribution made by the sugar and sugar derivative group to the number of carbon sources utilised at all time points. With

the exception of the 168 h biofilms, this group was the predominant group being utilised by the resident cells. The polymer group was also ever present throughout all the time points sampled, although no more than two polymers appeared to be utilised at any one time point.

What was also evident was the increase in utilisation of the differing functional groups by the microbiota after 24 h of incubation when compared to the 6 h incubation. The 72 h biofilms after 6 h incubation were metabolising substrates from 3 functional groups compared to 4 after 24 h of incubation. Indeed the number of functional groups being utilised at the 72 h sampling point after a 6 h incubation decreased when compared to the comparative data generated by the 24 h biofilm. The 168 h biofilms after 6 h incubation were using carbon sources from 5 functional groups compared to 6 after 24 h incubation. Hence it appears that the longer incubation time results in an increase in the number of carbon sources being utilised as well as a concomitant shift in the functional groups being metabolised.

6.3.1.2 Run 2

Figure 6.2: A bar chart showing the number of positive wells for both aerobic and anaerobic plates inoculated with biofilms from run 2, both 6 h and 24 h post inoculation.



6.3.1.2.1 The profile after 6 h incubation

Figure 6.2 shows the number of carbon substrates utilised during biofilm development from the second fermentor run after 6 hours and 24 hours

incubation of the microplates both aerobically and anaerobically. The 24 h biofilms after 6 hours incubation utilised 2 carbon substrates. Substrate utilisation increased to reach its maximum with the168 h biofilms, after 6 hours incubation, 20 positives were evident, including sugars, methyl esters, carboxylic acids, amino acids and peptides, alcohols and sugar phosphates. A decrease in the number of carbon substrates utilised by the 240 h biofilms was evident relative to the 168 h biofilms, interestingly the alcohol group was only utilised by the biofilm microbiota at both the 168 and 240 h sampling points. However, the 336 h biofilms were able to utilise 9 carbon substrates from four functional groups, more than were utilised at either the 24 or 72 h sampling points.

6.3.1.2.2 The profile after 24 h incubation

After 24 hours incubation in the microplates, carbon utilisation increased when compared to the data collated after 6 h of incubation. The number of carbon sources being metabolised reached its peak with the168 h biofilms, which after 24 hours incubation were able to utilise 45 substrates from 8 different functional groups, this then decreased through the 240 h biofilms to the 336 h biofilms. However the 336 h biofilms utilised a wider range of substrates (17) than either the 24 h (3) or 72 h (15) biofilms.

The increase in the number of substrates utilised associated with 24 hours of incubation was mainly due to a large expansion in the number of the sugars and sugar phosphates utilised from the 72 h sampling point onwards. Interestingly

the polymer group was being utilised after 24 h of incubation by biofilms from all the sampling points, this contrasts with what found after 6 h of incubation where the polymer group was not metabolised by any of the sampled biofilms.

6.3.1.3 Comparative analysis of run 1 and run 2

There were qualitative similarities in the CLPP profiles generated by the biofilms from both runs; however there were some observable differences. The 24 h biofilms from run 2 after 6 hours incubation utilised 2 carbon substrates differing from the analogous set of biofilms from run 1 which utilised a greater range of carbon sources, 12. The 168 h biofilms utilised the largest number of carbon sources from both run 1 and 2, indeed the utilisation 'fingerprints' generated by both run 1 and 2 are rather similar, bearing a resemblance to the statistical poisson distribution. The 336 h biofilms from run 2 produced 9 positives after a 6 h incubation whereas the corresponding data from run 1 did not produce a positive. However the data from both run 1 and 2 demonstrated that incubation for 24 h within the microwells lead to a burgeoning of the number of positives generated from within the sugar and sugar derivatives group. This particular group seemed to account for the differences in carbon source utilisation between the 6 and 24 h incubations.

6.3.1.4 Comparative analysis of cumulative optical denities

Table 6.2 catalogues the total optical density recorded for both the aerobically and anaerobically incubated plates, the total optical density was then normalised

(per 10^4 cells) for both the 6 and 24 hour incubations of the microplates for both runs. Table 6.2 shows that the total (gross) density increased with biofilm age during both run 1 and run 2, in addition at all sampling points the total optical density was greater after 24 hours than 6 hours incubation. However, when the gross figure was adjusted to compensate for the effect of cell density (see chapter 8) the normalised figure decreased with increasing biofilm age until the 168 h (0.001 for 24 h incubation, 168 h biofilms) sampling point was reached, after this the normalised figure then recovered somewhat (0.003 for 24 h incubation, 336 h biofilms) although this represented a fraction of the initial reading c.1/10 (0.027 for 24 h incubation, 24 h biofilms). Table 6.2 also details what was found throughout the course of run 2, the total optical density again increased at each successive sampling point, mirroring what was found during run 1. However the normalised figure decreased with increasing biofilm age until the 168 h biofilms (0.001 for 24 h incubation, 168 h biofilms), again in agreement with what was found during run 1, this represents an appreciable reduction on the initial reading produced by 24 h biofilms (0.058 for 24 h incubation). Again (as during run 1) the normalised density increased through the final two sampling points (0.008 for 24 h incubation, 240 h biofilms; 0.011 for 24 h incubation, 336 h biofilms) but did not recover the level seen initially with the 24 h biofilms. Overall both the raw and processed data generated from run 1 and 2 share a high degree of homology. Interestingly although the cumulative optical density generated by run 1 was bigger than the comparative data point generated via run 2, this situation was reversed when examining the normalised optical density.

Table 6.2: A table detailing the cumulative optical density for all 96 wells as measured at 590 nm for the both the aerobically and anaerobically incubated plates from both run 1 and 2, the optical density per 10^4 CFU was calculated to compensate for differing cell densities.

Biofilm Age (h)	Run	Total well OD (6 h)	Total well OD (24 h)	OD per 10^4 CFU (6 h)	OD per 10^4 CFU (24 h)
24	1	36.497	39.799	0.025	0.027
	2	25.142	27.086	0.054	0.058
72	1	51.975	74.961	0.008	0.012
	2	33.296	42.246	0.006	0.007
168	1	64.757	80.106	0.001	0.001
	2	43.575	59.002	0.0008	0.001
240	1	68.774	83.035	0.001	0.002
	2	50.2	55.602	0.007	0.008
336	1	73.917	96.95	0.002	0.003
	2	67.432	70.615	0.010	0.011

6.3.2 Results of the DGGE analysis

The development of the biofilm community in the CDFF model was analysed using the culture independent technique, DGGE. Figure 6.3 is the perpendicular gel that was cast and run to determine the appropriate denaturant gradient for optimal band resolution. Figures 6.4 and 6.5 are the parallel gels showing the detectable phylotypes (bands) present within the biofilm community at each sampling point during the course of both runs.

Figure 6.3: The sigmoidal curve produced after two rounds of PCR followed by resolution upon a perpendicular gel is shown below.

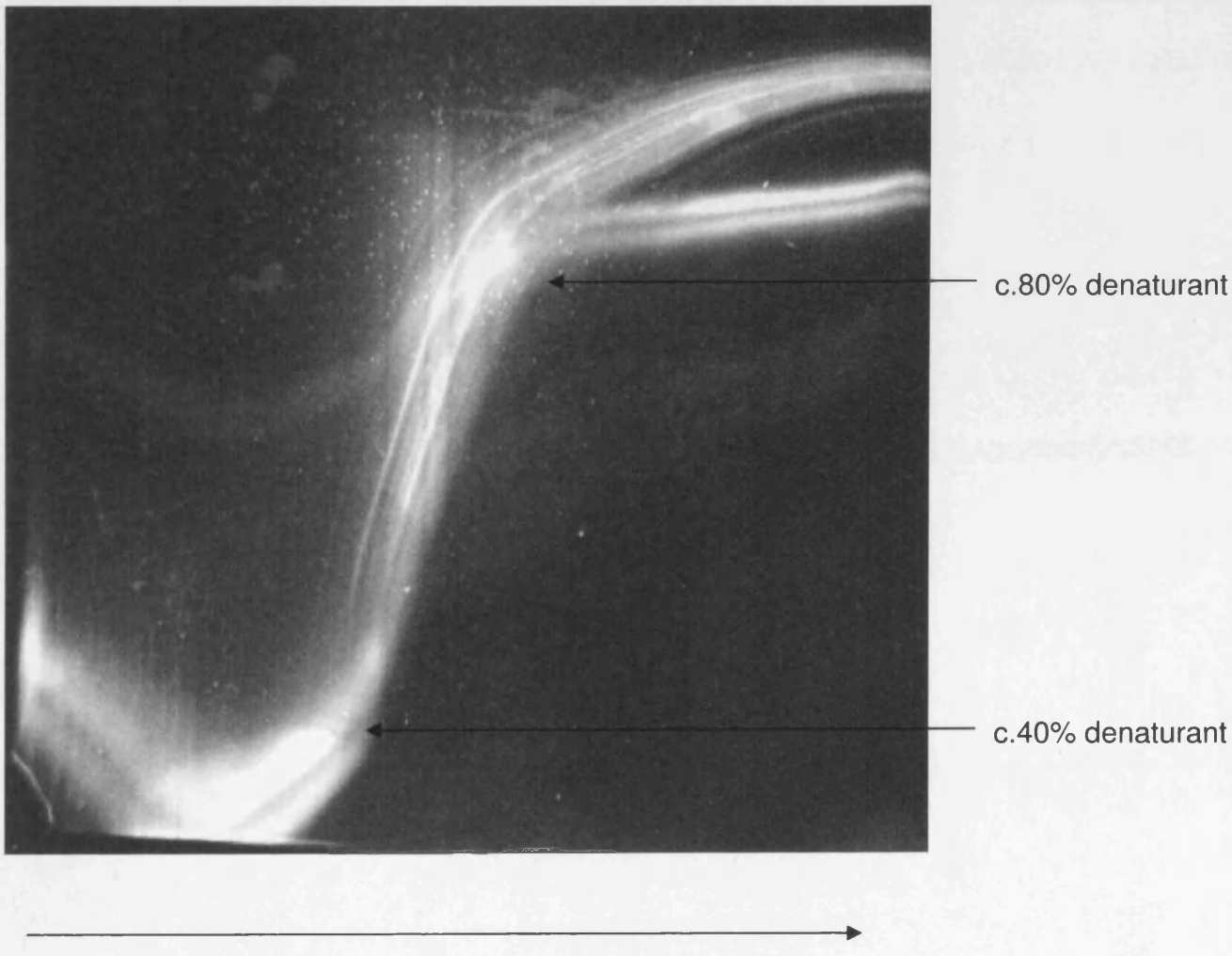
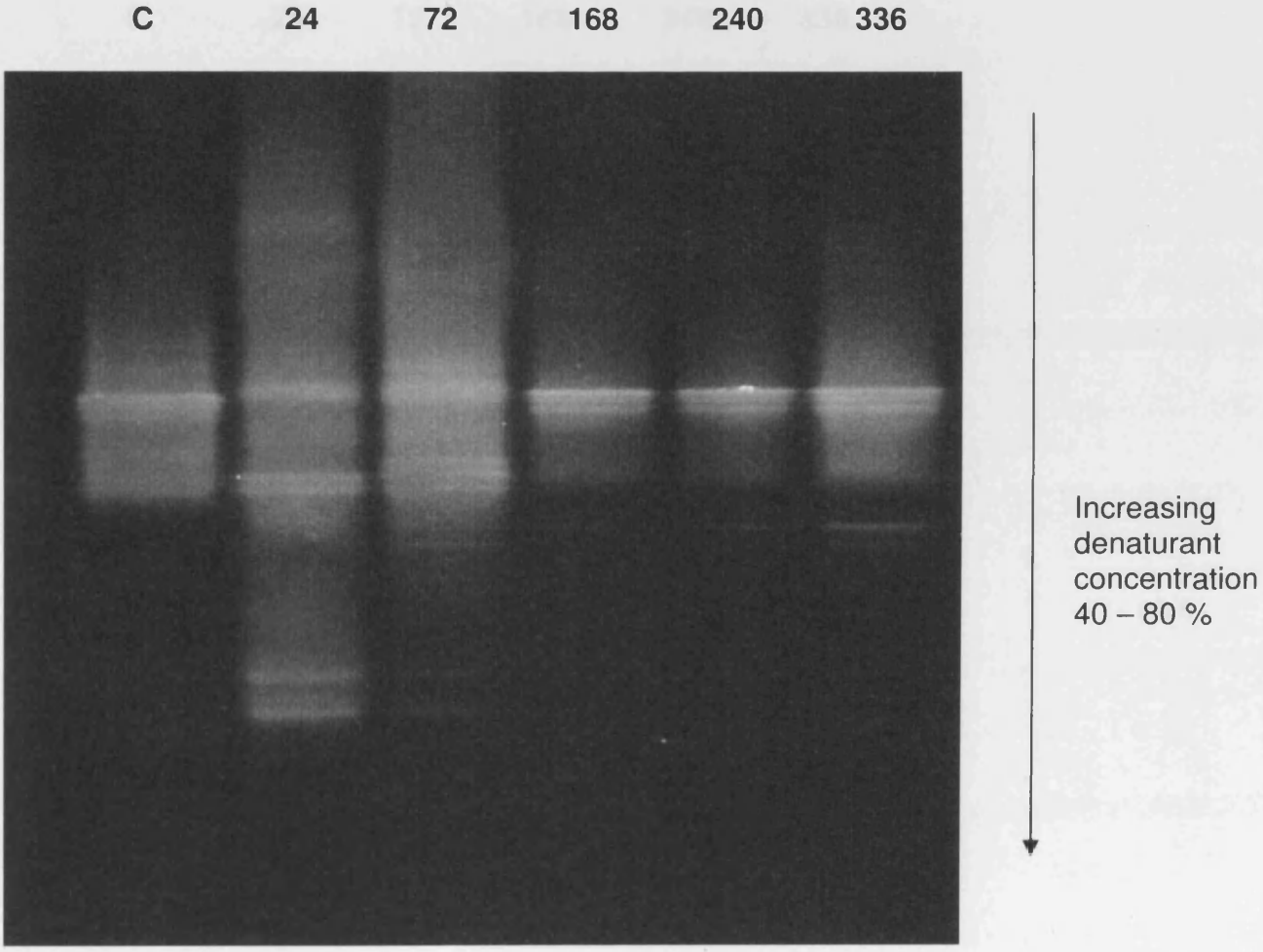


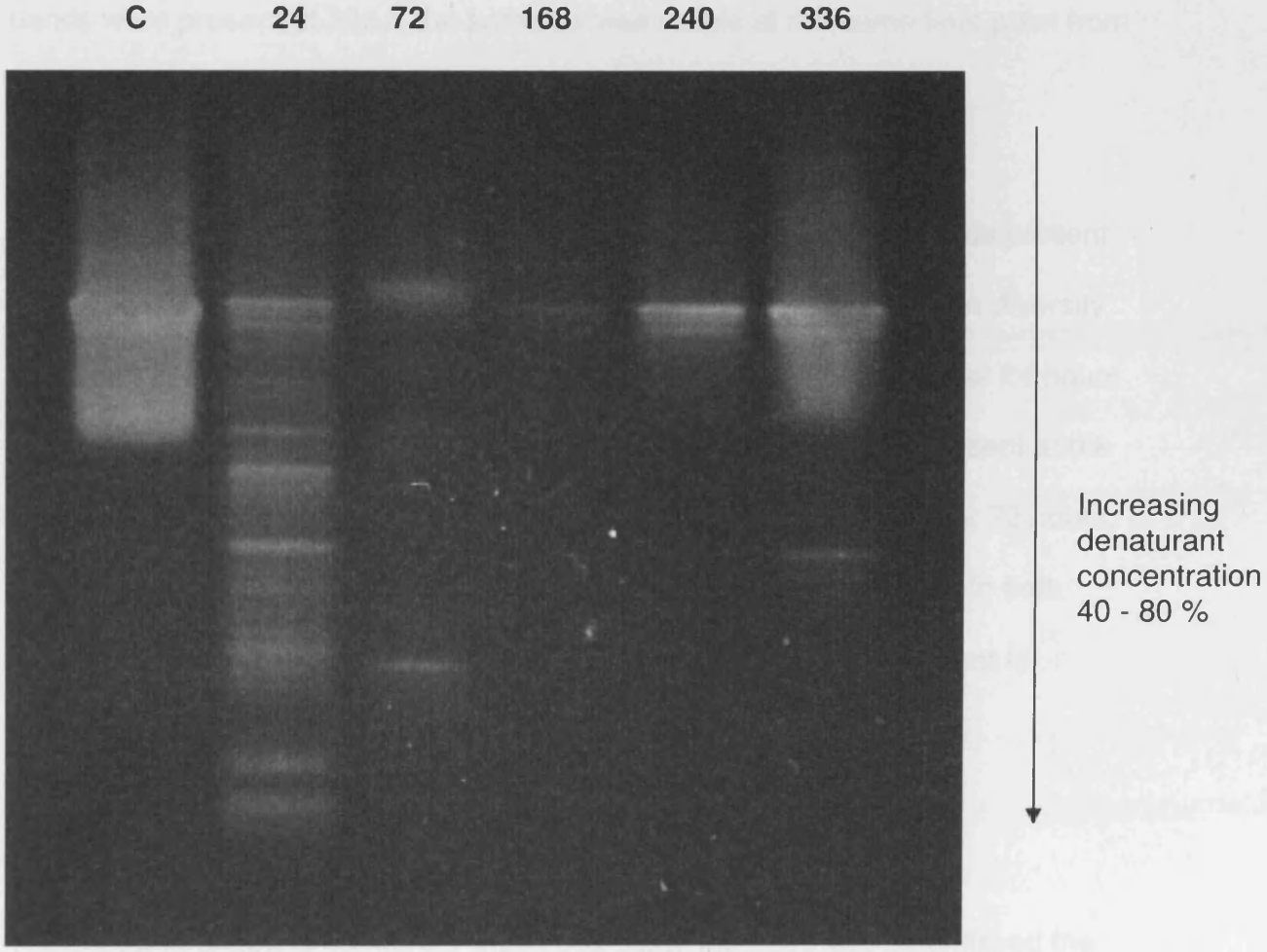
Figure 6.3 shows that optimal band resolution may be achieved using a 40 – 80 % denaturant gradient for the population of partially amplified 16S rRNA gene fragments generated by the biofilm microbiota; to optimise band resolution when running the parallel gel it was necessary to establish the melting behaviour of the DNA fragments and optimise the denaturant gradient (Muyzer at al., 1998).

Figure 6.4: The banding pattern produced by DGGE is shown in figure 6.4 after two rounds of PCR amplification from the biofilms taken at various time intervals from run 1. Lanes: C, a control which was *Porphyromonas gingivalis;* 24 was the banding pattern produced by 24 h biofilms, 72 by 72 h biofilms, 168 by 168 h biofilms, 240 by 240 h biofilms and 336 by 336 h biofilms.



The bands were enumerated for each time point, the 24 h biofilms produced 9 bands, this increased to 12 for the 72 h biofilms. The number of bands then decreased to 8 bands at 168 h, and 7 bands at both 240 h and 336 h sampling point.

Figure 6.5: A DGGE gel showing the banding patterns produced after two rounds of PCR amplification from the biofilms taken at various time intervals from run 2. Lanes: C, a control which was *Porphyromonas gingivalis;* 24 the banding pattern produced by 24 h biofilms, 72 by 72 h biofilms, 168 by 168 h biofilms, 240 by 240 h biofilms and 336 by 336 h biofilms.



The banding pattern from the biofilms taken from the second fermentor run is shown in figure 6.4. The bands were enumerated for each time point so the 24 h biofilms produced 20 bands, this decreased on progression to the 72 h biofilms where 7 bands were evident. The number of bands then decreased to only 2 bands at 168 h, the number of bands then increased to 5 bands at 240 h, 7 bands were present at 336 h the same as was visible at the same time point from run 1.

What was apparent from both DGGE profiles was the number of bands present initially was reduced as the biofilm matured, this reduction in phylotype diversity was most distinct within run 2, but was also evident during run 1 . After 24 hours during run 2, 20 bands were present, substantially more than was present at the corresponding time point during run 1, this was then reduced to 7 after 72 hours, less than was apparent at the corresponding time point during run 1. In both runs however, after 336 hours, the same number of bands were evident at approximately the same migratory distance on the gel.

6.4 Discussion

The previous chapters contained within this study thus far have established the efficacy of TBO in conjunction with HeNe laser light in killing planktonic oral organisms, even when those organisms compose both mono and multi-species biofilms. The biofilms which have been previously generated in the course of this study were done so using a non steady-state system, inoculation of membrane

filters which were placed upon agar plates was a simple methodology which permitted rapid screening against a range of differing organisms. However to produce biofilms that mimicked those associated with periodontal disease *in vivo,* a more sophisticated approach was necessary using a model that permitted the closer approximation of both the biotic and abiotic conditions *in situ* within a periodontal pocket, the disease lesion within which the aetiological agents of periodontal disease, the periodontopathogens may be isolated (Greenstein et al., 2001). The CDFF which has been described previously (see section 1.4.1 and 2.2) is known to be amenable to modelling the oral cavity as it has previously been used to successfully model supragingival dental plaque (Wilson et al., 1995b, Pratten et al., 1998b, Pratten and Wilson 1999, Roberts et al., 1999). The medium used in this study consisted of both RPMI and horse serum, RPMI is used to culture eukaryotic cells in tissue culture hence its composition was formulated to ensure its compatibility with body fluids. The serum concentration used was similar to that found in the GCF of healthy individuals (Cimasoni 1983, Curtis et al., 1988). Indeed it has previously been demonstrated that serum is suitable for the growth and maintenance of a pathogenic subgingival microbiota (Ter Steeg et al., 1988). Hemin and menadione were added to the nutrient medium to simulate the effect of bleeding which is known to occur with inflammatory periodontal diseases (Jorgensen et al., 2001). The gaseous environment in the CDFF when cultivating the biofilms was microaerophilic, periodontal pockets are known to have a low redox potential (Marsh., 2003), this is often dependent on the depth of the disease lesion which itself is dependent

upon the severity of the disease state. Due to the factors mentioned the level of oxygenation varies from one periodontal pocket to another, hence the 2 % O_2 which was added to the CDFF was based upon a mean value, taken from several studies, of the oxygen content within periodontal pockets (Loesche et al., 1983, Mettraux et al., 1984).

In the present study the metabolic and genetic diversity of the microbial community generated within the CDFF from an inoculum taken from pooled homogenised periodontal disease lesions *in vivo* was investigated. DGGE and CLPP have been shown to be useful methodologies in resolving interactions and bacterial activity within complex microbial communities, both of these methods have been used previously to determine the effect of perturbation upon microbial communities and indeed to monitor microbial community development. CLPP was used in this investigation to monitor the growth and successional events which occur as a biofilm accumulates and matures. CLPP has been extensively used for this purpose previously and was thought to be an appropriate method for determining changes and vacillation in the composition of the resident microbiota within the confines of the biofilm.

The CLPP analysis of metabolic diversity within the microbial communities produced some interesting data, the total number of carbon sources utilised by the biofilms from both CDFF runs varied somewhat, there were, however, some identifiable trends that were apparent in both runs. Both the aerobic and

anaerobic microplates were analysed 6 h and 24 h post-inoculation and the number of positive wells relative to the control determined, generally it appeared that incubation of the plates for 24 h did not produce any meaningful additional data, the biofilm cells simply appeared to grow and divide within the media containing the easily metabolisable sugars and sugar derivatives, inflating the number of positives from within this functional group of carbon sources, indeed it has previously been acknowledged that conditions within the microwell actively select for fast growing bacteria that are impervious to the effect of tetrazolium salt (O'Connell et al., 2000). Perhaps the somewhat shorter incubation time (6 h) gave a better indication of the metabolic diversity inherent within the microbial communities. Indeed the longer incubation time within the microwells may have simply provided the inoculated community with sufficient time to not only divide but also adapt to the prevailing conditions, allowing the cells to switch on the appropriate operons permitting the cells to fully exploit the carbon source (Kresse et al., 2000). Hence what was being observed after 24 h of incubation was the response of the biofilm cells to changes in the prevailing environmental conditions, and not an accurate profile of what was occurring within the confines of the biofilm at the time of harvesting from the model. Indeed Haack et al, 1995 noted that when employing CLPP for community analysis careful monitoring of colour development was required so that the effects of any nonlinearity in substrate utilisation patterns may be accounted for (although this was not easy to implement in practice due to the number of concurrent experiments being conducted).

Certainly whist CLPP has been used for community analysis previously many authors refer to problems with the use of carbon utilisation as a 'fingerprinting' methodology. Smalla et al., 1998 used TGGE to analyse the diversity present within the BIOLOG GN plates 48 h post-inoculation with cell suspensions from a potato rhizosphere, compared to the diversity present within the initial inoculum. The authors found that there was a decrease in diversity within the microwells of the BIOLOG plates relative to the diversity of the initial inoculum. Moreover the composition had been perturbed such that species which had been numerically dominant in the inocula where not detected in some of the BIOLOG wells. It should be noted that in the confines of this study the microplates were read 6 h and 24 h post inoculation of the plates, hence the flaws outlined by the Smalla et al., study are not directly applicable, although one may hypothesise that a certain perturbation of the original inocula may have occurred, certainly after 24 h of incubation. Perhaps the increase in the number of positives from the sugar and sugar derivatives functional groups after 24 h of incubation may have been due in part to a modicum of community perturbation, or perhaps due to the methods employed for sample preparation.

The biofilm structure was homogenised to allow sample inoculation. As discussed previously (see introduction) growth as a biofilm introduces gradients of nutrient availability throughout the z – plane of the biofilm sample homogenisation would instantly relieve such constraints on nutrient availability for cells that previously may have been encased within a biofilm microcolony. Thus

utilisation of carbon compounds available in a microwell after a period of adaptation to the new prevailing physiological conditions may have occurred, such adaptation would have been time dependent, hence a 24 h incubation may have provided a sufficient time window for this process to occur. Perhaps what was being observed after a 24 h incubation was adaptation to the prevailing conditions, to an environment where gradients simply did not exist. In addition, there is also a known correlation between initial inoculum microbial density and the rapidity of well colouration (Haack et al., 1995). It is known that cell and hence inoculum density increased as a function of time (see chapter 8). This undoubtedly would have had a knock-on affect upon the CLPP profile although this inoculum dependent effect was corrected for via the use of a normalised cumulative total BIOLOG plate optical density.

What also remained unresolved was the relative role of all members of the community in the production of the metabolic profile at each time point. What was interesting was that the normalised cumulative optical density decreased as a function of time up until the 168 h time point, this trend was evident in both runs. Indeed the biofilms extracted at the 168 h sampling point produced the lowest normalised total optical density, in addition, at this time point, in both runs, the largest number of CLPP positives was recorded, (i.e. those wells in excess of 140% of the control.). Therefore one could postulate that at the 168 h time point the cells were not as metabolically active as they had been at the earlier sampling points as revealed by the progressive decrease in the normalised OD,

despite the highest viable counts being recorded at this particular sampling point (see chapter 8). It appears that as the biofilm matured, metabolic capacity per bacterium decreased, however this effect was more than compensated for by the increase in cell numbers masking the putative decrease in metabolic dynamism of each community constituent. Interestingly during both runs the normalised OD recovered somewhat over the course of the remaining two sampling points (240h and 336 h), although the reasons for this are not immediately apparent, this did correlate with a progressive decrease in the viable count recorded over the course of the final two sampling points (see chapter 8).

Other interesting questions remain which could not be answered entirely satisfactorily, such as was the CLPP profile a result of synergy between members of the microbiota. Haack et al., (1995) concluded that they found no evidence to suggest synergism between isolates in kinetic analyses. Additionally it was hard to ascertain whether all the cells capable of utilising the carbon substrates contributed to the generation of the profile (Haack et al., 1995). However the diverse range of carbon sources available, unlike typical homogenous microbial media, would have produced a large number of differing niches, allowing microbial species which may not have survived using traditional cultivation techniques to proliferate and contribute to colour formation (Smalla et al., 1998). Conversely, the requirement for growth may have resulted in enrichment, particularly those cells which may react rapidly to high substrate concentrations i.e. copiotrophs (Garland, 1997) this effect would have been

rather more pronounced with the 24 h incubation. What was evident was the need for a conservative interpretation of the data generated by CLPP, one must bear in mind that any conclusions reached must be qualified by the outlined limits inherent within this profiling technique. Indeed the BIOLOG system has been employed to gauge the metabolic activity occurring within oral bacteria communities previously (Anderson et al., 2002). The authors concluded that BIOLOG CLPP analysis does provide useful data as to the metabolic potential of the constituent bacteria composing the biofilm, with the important proviso that incubation conditions may unduly affect the subsequent profile. That conclusion is supported by the data that have been generated in this study; the results obtained demonstrate that incubation conditions do indeed influence the resultant CLPP profile. Hence it did appear that the metabolic potential of the microbial community increased as a function of time up until 168 h post-inoculation of the fermentor, whereupon it then decreased. However, whilst the metabolic activity of the community increased as the biofilm matured, this corresponded with a decrease in the metabolic activity of the composite microbiota. Hence, whilst it appears that community metabolic potential increased to its zenith at the 168 h sampling point and then decreased, the inverse seems to be true when one examines the metabolic activity per bacterium. One may postulate as to why this may have been the case. Upon the onset of biofilm formation the pioneer cells are rapidly utilising the available nutrient sources and concomitantly dividing, utilising the available carbon sources, without the inhibitions of vigorous competition for nutrients and the relative absence of metabolic waste products or

secondary metabolites. As the biofilm matured metabolic activity may have been limited via the presence of secondary metabolites, increased competition for metabolites or due to diffusional impediments such as bacterially derived EPS, decreasing the metabolic activity of the biofilm microbiota. The recovery of per bacterium metabolic activity may have been due to decreased competition for nutrients after the 168 h sampling point (see chapter 8). In addition, it would be hard to envisage that a degree of enrichment did not occur during incubation of the microplates, resulting in the increase in the number of positives within the sugar functional group after 24 h of incubation. Therefore it would appear that the 6 h incubation provides a better insight into the metabolic potential of the biofilm community at the sampling point, perhaps a better 'snapshot' of metabolic potential instead of determining phenotypic plasticity. The corollary of this was perhaps CLPP is best suited to the analysis of gross metabolic and hence potential compositional changes within a community, Ellis et al., 2001 came to the same conclusion regarding CLPP when it was employed to determine the effects of heavy metal contamination upon soil communities, any slight perturbation or shift within the community may not have been accurately reflected in the resultant CLPP fingerprint.

DGGE has recently been applied to analyse the inherent diversity present within a periodontal pocket (Zijinge et al., 2003) this research is pertinent as the inocula used during the course of the two runs were derived from biological material extracted from inflamed periodontal pockets. The study involved the extraction of

samples from subgingival pockets from patients receiving treatment as well as from periodontally healthy family members, 12 samples taken from four patients were evaluated for shifts in community structure one day and subsequently three months post-treatment. If the data are interpreted semi-quantitatively the treatment resulted in a reduction in the overall bacterial richness as represented by a reduction in the number of observable bands. This conclusion was qualified by the authors who stated that the subgingival environment is prone to shifts in composition and diversity that are not always readily explainable and may occur independently of any perturbation. Zijinge et al., (2003) employed DGGE as a relatively rapid method that permitted the genetic diversity present at a particular time point to be analysed, hence the technique is amenable to multiple sampling facilitating a comparative analysis making it well suited to analysing community structure over time. DGGE has also been utilised to study community development over time within a CDFF (McBain et al., 2003). This study validated the use of DGGE as an important tool when attempting to determine the relative stability of community structure over time. DGGE also revealed that substantial differences were evident in the composition of the inoculum when compared to the microcosm communities that became established within the CDFF, the conclusion being that without the application of DGGE these data would not have been available. Hence DGGE was employed as part of this study due to its inherent utility and the relative rapidity aptly demonstrated in the two investigations outlined, providing data that would not be otherwise available.

The characterisation of the development of the microbial community within the CDFF produced some interesting data, the broad conclusion that was drawn from the data generated by both runs was that the initial diversity present at the earliest sampling points decreased as a function of time, the supposition being that the diversity that was present within the inoculum was not maintained within the CDFF over time on both occasions, despite the CDFF being set-up to closely mimic both the biotic and abiotic conditions prevalent within a periodontal pocket. Hence 24 hour biofilms, in run 2, resulted in a DGGE profile with 20 bands, this was then reduced to 7 bands present at the final sampling point after 336h. This situation was reflected in run 1 where initially after 24 h 9 bands were evident in the DGGE profile, but ultimately after 336 h 7 bands were evident at approximately the same electrophoretic distance as the 7 bands that were evident at the corresponding time point from run 1. The McBain et al., (2003) study concurred with this conclusion, stating that DGGE revealed dynamic-change occurring within a putatively steady state system as determined by more traditional cultural analysis. The inoculum used in both runs was composed of pooled homogenised periodontal plaque, each run inoculated from the same batch of pooled plaque. Hence the subsequent community which developed within the CDFF was a microcosm, this has been defined as "a laboratory subset of a natural system from which it originates but from which it also evolves" (Wimpenny., 1988). The DGGE results suggested that the diversity represented within the inoculum was not evident within the CDFF. It was undetermined how diverse the original inoculum was. However by extrapolating past the 24 h

sampling point for which there was a banding pattern and acknowledging the known diversity of the subgingival environment c.500 species (Paster et al., 2001) it would be reasonable to suggest that the inoculum was indeed more diverse than the biofilm communities sampled from the CDFF. Certainly it has been previously shown that microcosm composition within the CDFF may vary considerably from the composition of the original inoculum (McBain et al., 2003). Indeed this paradigm of diversity reduction upon growth within the CDFF has been reflected upon before, Pratten et al., (2003) cultivated a supragingival microcosm within the CDFF and concluded that the diversity present within the CDFF was not a true reflection of the *in vivo* situation, in that instance conditions within the model were implicated as possible reasons for a decrease in species diversity. Certainly the model which was used to approximate the subgingival environment was not perfect, as indeed are all *in vitro* models in that they lack the incorporation of a host response. Biofilm formation and composition is likely to be highly influenced by interactions with the host, particularly the host immune system. For example secretory IgA, the predominant immunoglobulin in the oral cavity, can agglutinate bacteria, modulate enzyme activity and inhibit adherence to epithelial cells, conversely it may also act as a bacterial nutrient source (Reindholt and Kilian., 1987). One may postulate that whilst complex interactions with the host may contribute to conditions conducive to growth, the host may also inhibit bacterial growth, certainly via the immune system. Perhaps the influence of the host upon the direction of biofilm development *in vivo* may

have to be taken into account when developing the next generation of *in vitro* models.

The DGGE technique is somewhat limited in that bacterial populations which constitute less then 1 % of the total community may not be detected (Muyzer et al., 1993). Estimations of species composition may be skewed due to PCR bias which may result from primer bias due to the use of a certain set of 'universal' primers or relating to the number of rounds of PCR used, both of these have been shown to contribute to the sensitivity of PCR previously (Pratten et al., 2003). DGGE has proven to be a versatile and adaptable technique and has been widely employed by environmental microbiologists in a number of theatres where bacterial populations are perceived to be in a state of flux. Miambi et al., (2003) utilised DGGE to determine representative bacteria involved in the fermentation of cassava dough. In a similar application Randazzo et al., (2002) employed DGGE to determine shifts in the bacterial populations that occurred concomitantly with Sicilian cheese manufacture. This allowed the complex shifts in population to be accurately monitored giving feedback useful in the production of starter cultures. Interestingly, it was found that the initial microbiota found within the unprocessed milk was very different to the microbiota present within the final ripened cheese, this result is akin to what was found in the present study where a palpable change in the predominant phylotypes was evident over time.

The DGGE analysis that was carried out indicated there was a decrease in species diversity over time, however this should be qualified. The DGGE analysis presented within this chapter represents what might be best described as an initial foray into determining the diversity present within the CDFF and how this varied over time. To fully optimise the assay, the bands would have been excised from the gel and sequenced to determine the species which predominated, the PCR amplification could then incorporate primers for the bacterial groups that predominated, hence improving the resolution of the technique. Okabe et al., 2002 used a combination of fluorescence *in situ* hybridisation (FISH), the incorporation of specific primer pairs along with universal PCR primers and microelectrode measurements of oxygen, hydrogen sulphide content as well as the pH to study succesional developments in populations of sulphate reducing bacteria. This polyphasic approach permitted a thorough analysis of succesional developments and the physical distribution of the cells within the biofilm to be determined as well as the measurement of certain atmospheric indicators to be monitored.

The results of this investigation have shown that it was possible to cultivate biofilms derived from periodontal plaque within the CDFF. CLPP analysis of the biofilms revealed that the metabolic potential of the biofilms generated fluctuated over time but maximal metabolic plasticity was reached after 168 h of biofilm cultivation. However due to the outlined limitations in the methodology the results need to be interpreted cautiously. The DGGE analysis showed that initial

diversity that was apparent was reduced over time to a stable, yet less diverse community after 336 h of growth. In the following two chapters, the biofilms which were cultivated during the two runs will be analysed using different more traditional methodologies. However, by using a wide range of analytical methods, it is hoped that that this polyphasic approach may contribute toward a fuller understanding of biofilm development within the CDFF.

CHAPTER SEVEN

MICROSCOPIC ANALYSIS OF CDFF CULTIVATED SUB-GINGIVAL BIOFILMS

7.1 Introduction

Biofilms were cultivated within the CDFF and analysed at the community level in the previous chapter at the community level using techniques which have been utilised extensively to produce both metabolic (CLPP) and genetic (DGGE) fingerprints. There are, however, a number of other techniques, some of which are regarded as somewhat more traditional, which may be employed to gather information on the development of biofilms both *in vitro* and *in vivo*. The previous chapter was concerned with the composition of the entire community and how this fluctuated as a function of biofilm development and hence time. To complement the previous chapter, maintaining the community centric analysis, microscopic techniques were used to provide a visual reference of biofilm development of the very same batch of biofilms as analysed in the previous chapter. Hence in using Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM), biofilm development was observed and recorded over time within the CDFF, allowing comparisons to be made between biofilm development within the model (CDFF) and of plaque development *in vivo*, which has also been described using these same microscopic techniques.

7.2 Materials and Methods

The CDFF was set up, inoculated and sampled as described in chapter 6, section 6.2

7.2.1 Confocal laser scanning microscopy (CLSM) of biofilms

This was carried out as described in chapter 2 section 2.5

7.2.2 Transmission electron microscopy (TEM) of biofilms

Discs with attached biofilm were fixed in 3 % glutaraldehyde solution, 0.2 % ruthenium red in 0.1 M sodium cacodylate buffer at 4 °C overnight. The specimens were post-fixed in 1 % osmium tetroxide at 4 °C for 2 h followed by dehydration in a series of alcohol concentrations (20 – 100 %, 15 minutes application per concentration). The specimens were then embedded in araldite CY212 and sections cut (section thickness varied dependent on biofilm depth). The biofilms were stained with lead citrate and uranyl acetate, the sections were viewed with a Jeol 100 CX transmission electron microscope (Jeol Ltd, Welwyn Garden City, U.K).

7.2.3 Scanning electron microscopy (SEM) of biofilms

Discs with attached biofilm were fixed in 3 % glutaraldehyde in 0.1 M sodium cacodylate buffer at 4 °C overnight. The specimens were then post-fixed and dehydrated as above in a graded series of alcohol concentrations. The specimens were then rinsed 3 times (10 minutes per rinse) in 100 % acetone, immersed in hexadimethylsilane for 1 – 2 minutes and left to dry in a dessicator. The specimens were viewed with a Cambridge 90B Stereoscan electron microscope (Cambridge scientific instruments Ltd, Ely, U.K).

7.3 Results

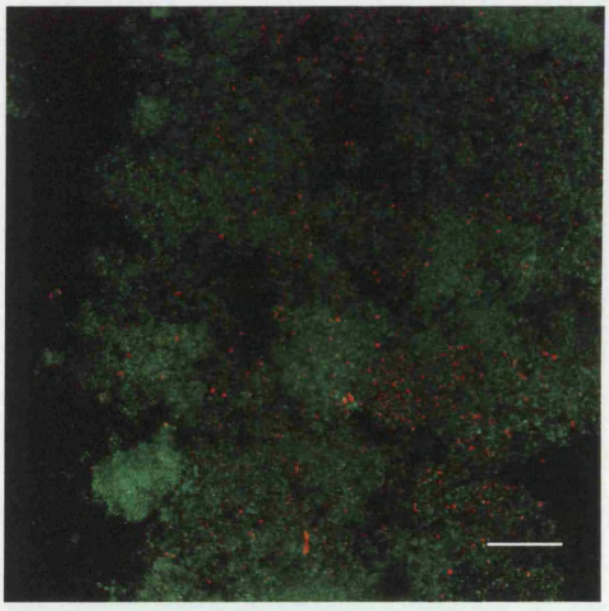
Biofilms were cultivated on hydroxylapatite discs in a CDFF. After 24, 72, 168, 240 and 336 h, biofilms were removed and prepared for visualisation in a manner dependent upon the technique being used.

7.3.1 Confocal microscopy of biofilm development

The confocal micrographs, figures 7.1 and 7.2, revealed how the number of viable cells relative to the number of non-viable cells fluctuated within a developing microcosm biofilm. Indeed, locally abundant coverage of cells upon the hydroxylapatite substratum was apparent after 24 h of growth.

The confocal micrographs, figures 7,1 and 7.2, revealed how the number of viable (green) cells relative to the number of non-viable cells (red) fluctuated within a nascent biofilm. What was also apparent was the locally abundant coverage of cells upon the hydroxylapatite substratum after 24 h of growth.

Figure 7.1 A confocal micrograph of a 24 h biofilm, the bar represents 20 µm



In figures 7.3 and 7.4 qualitatively there Appeared to be a higher proportion of viable compared to non-viable cells within these 72 h biofilms, certainly the biofilm appeared to be denser than was evident with the 24 h confocal micrographs. Indeed there appeared to be a reduction in the number of non-viable cells present compared to that which was observed at the 24 h sampling point

Figure 7.2: A confocal micrograph of a 24 h biofilm, the bar represents 20µm

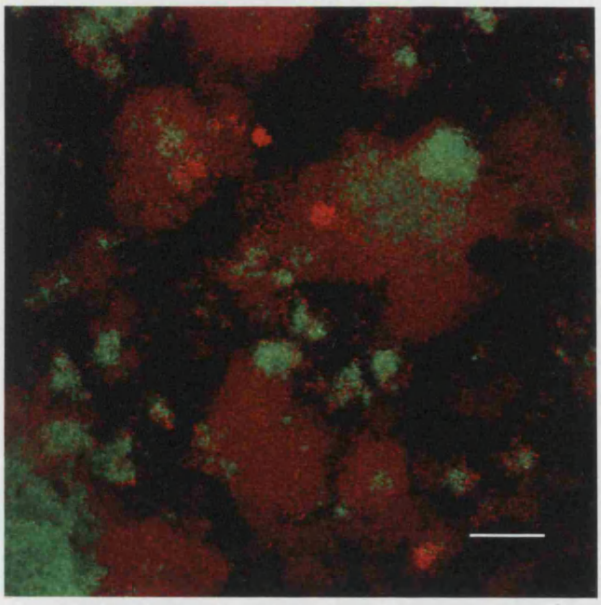


Figure 7.3: A confocal micrograph of a 72 h biofilm, the bar represents 20 µm

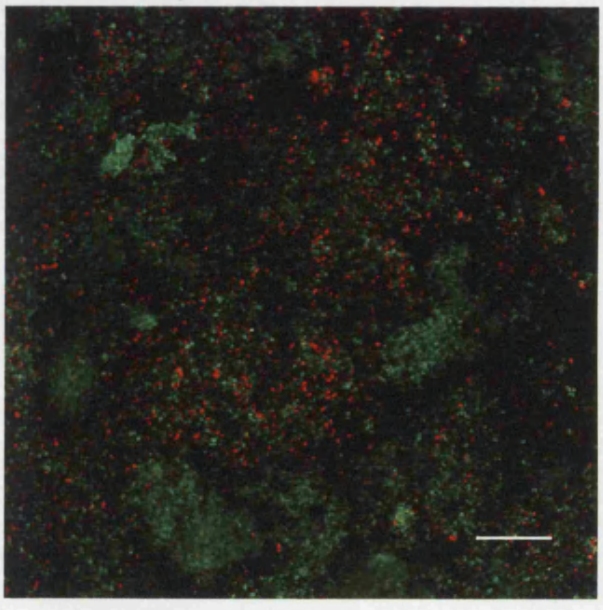


Figure 7.4: A confocal micrograph of a 72 h biofilm the bar represents 20 µm

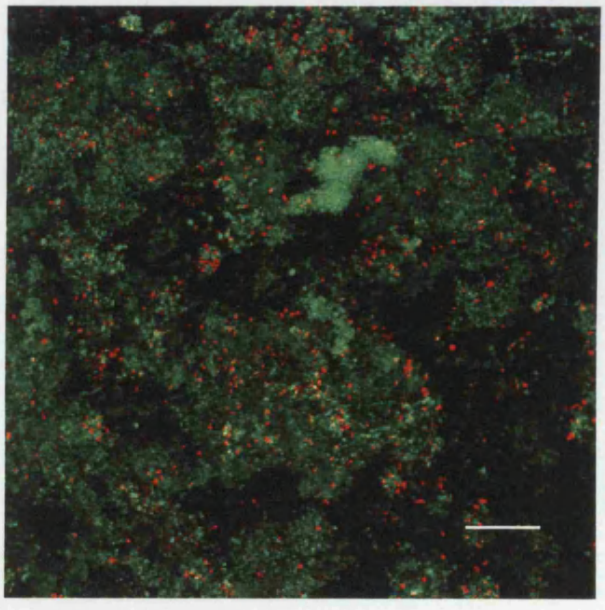
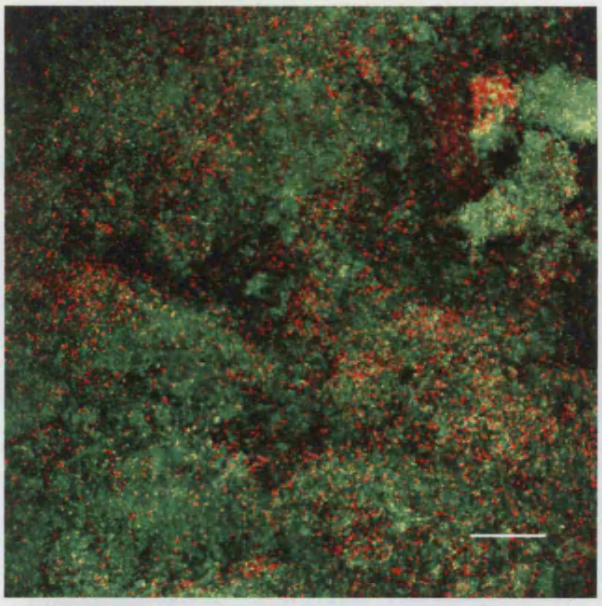


Figure 7.5: A confocal micrograph of a 168 h biofilm, the bar represents 20 µm



Both Figures 7.5 and 7.6 revealed a high proportion of viable to non-viable cells within the biofilm after 168 h of growth within the fermentor. Qualitatively the biofilm was somewhat denser compared to what was seen at both 24 h and 72 h.

Figure 7.6: A confocal micrograph of a 168 h biofilm, the bar represents 20 µm

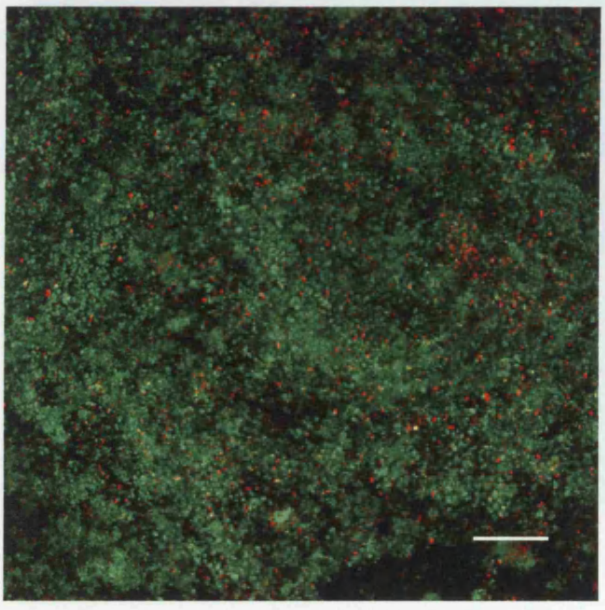


Figure 7.7: A confocal micrograph of a 240 h biofilm, the bar represents 20 µm

Figures 7.7 and 7.8 were confocal micrographs of 240 h biofilms. Within the micrographs were a large number of both viable and non-viable cells. The structural motifs associated with mature biofilms such as tower formations were identifiable within figure 7.8.

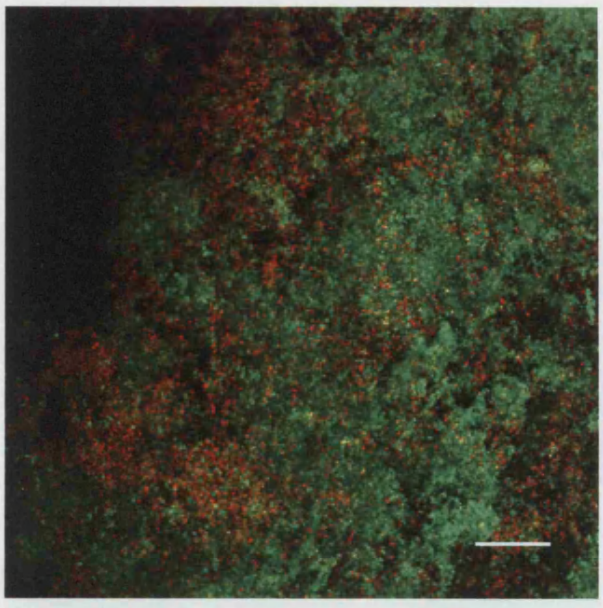


Figure 7.8: A confocal micrograph of a 240 h biofilm, the bar represents 20 µm

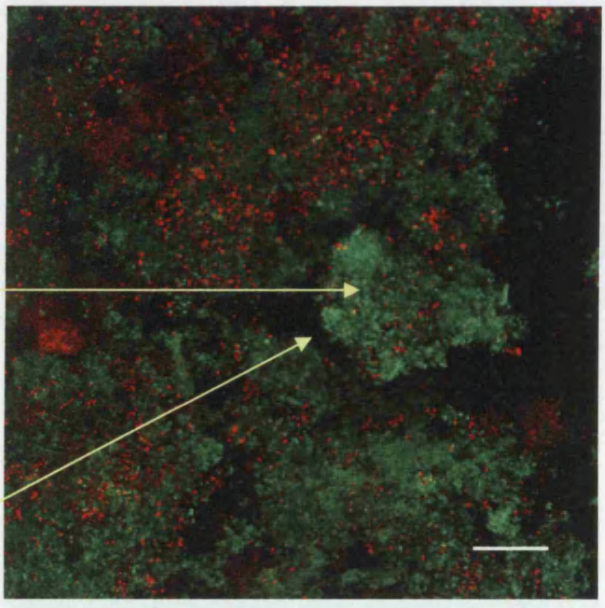
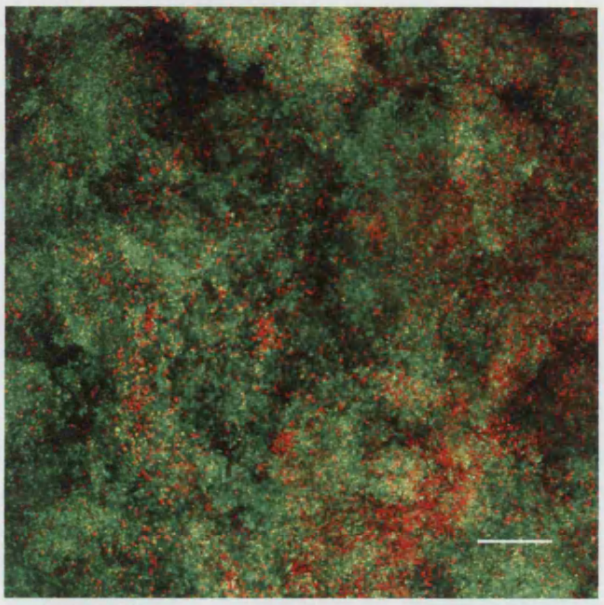
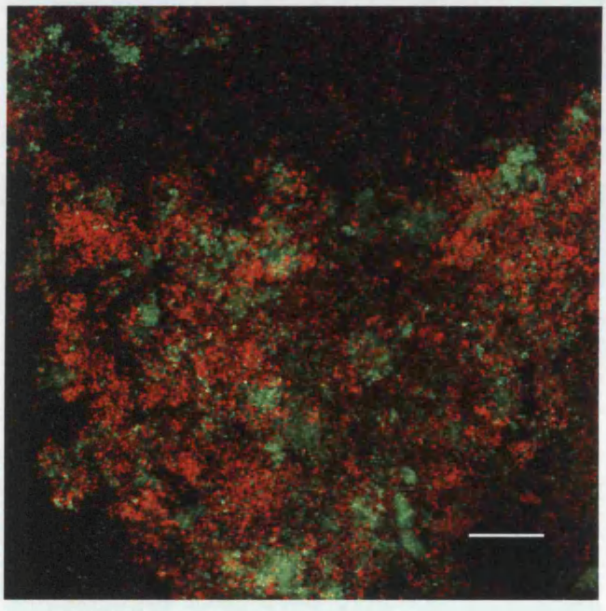


Figure 7.9: A confocal micrograph of a 336 h biofilm, the bar represents 20 µm



Both in figures 7.9 and 7.10 there appeared to be a qualitative increase in the proportion of non-viable to viable cells compared with the 240 h biofilms. The three-dimensional structure of the biofilms appeared to be present, perhaps not as defined as seen previously. Indeed cellular viability appeared to be waning.

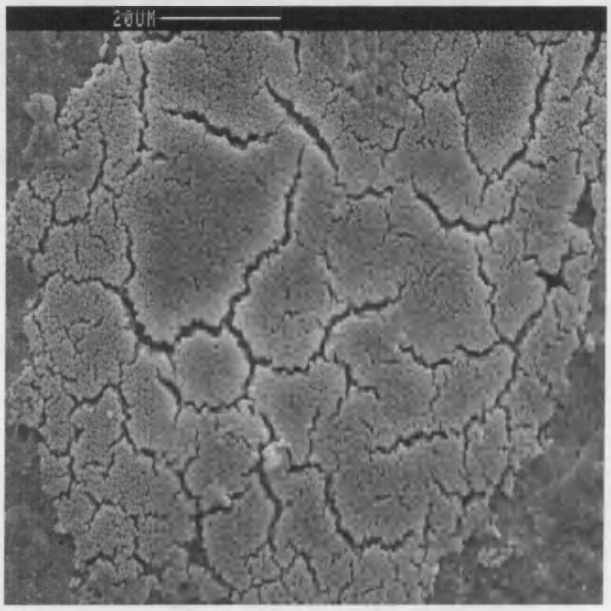
Figure 7.10: A confocal micrograph of a 336 h biofilm, the bar represents 20 µm



7.3.2 Scanning electron micrographs showing biofilm development

The CLSM data proved useful for qualitative determinations as to the ratio of viable to non-viable cells within the biofilm. SEM, whilst not permitting the visualisation of the biofilm in its hydrated native state, provides images which revealed morphotype diversity within the biofilm, and how this varied with biofilm age.

Figure 7.11: A SEM image of a 24 h biofilm



Figures 7.11 and 7.12 revealed how sparse the coverage of the substratum was after 24 h of growth within the CDFF. Qualitatively, the microbiota appeared to be dominated by cocci which were evident in both electron micrographs.

Figure 7.12: A SEM image of a 24 h biofilm

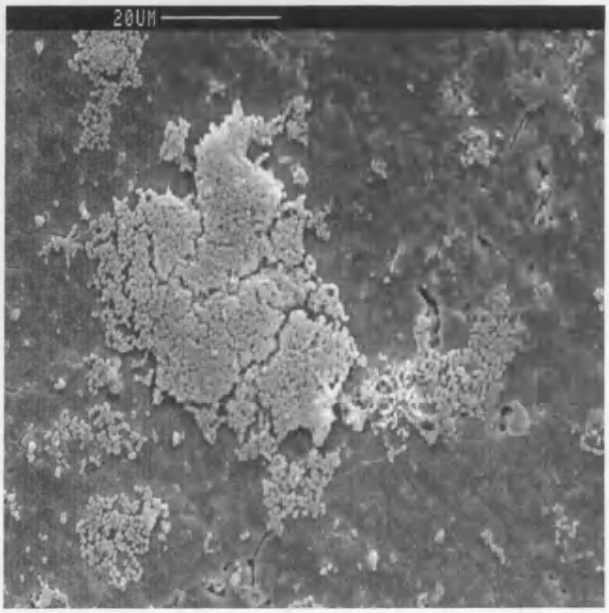
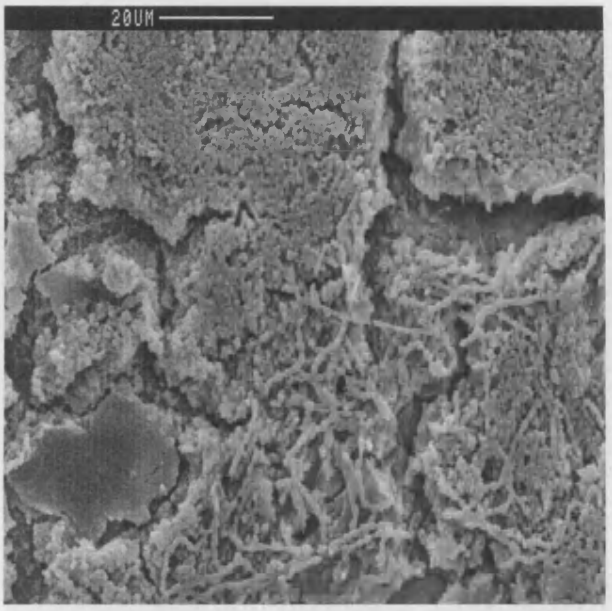


Figure 7.13: A SEM of a 72 h biofilm



Figures 7.13 and 7.14 revealed that the biofilms appeared to be exhibiting increased diversity, long chains of cocci were evident; these were not present after 24 h of biofilm growth. However the vast majority of the cells evident within the micrographs still appeared to be coccoid.

Figure 7.14: A SEM of a 72 h biofilm

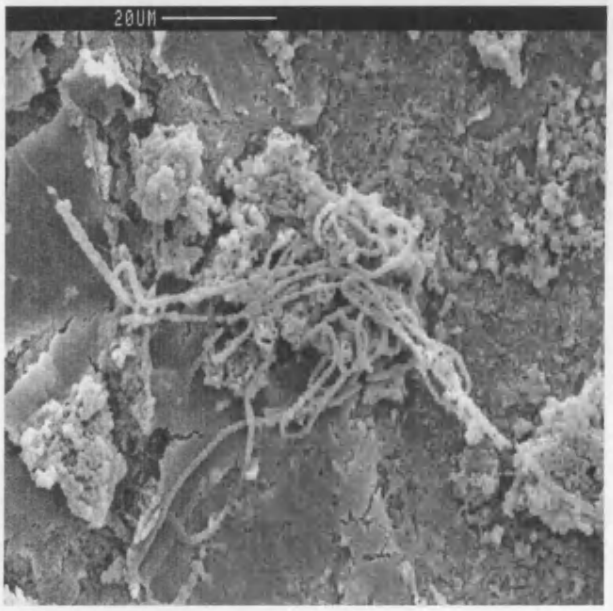


Figure 7.15: A SEM of a 168 h biofilm

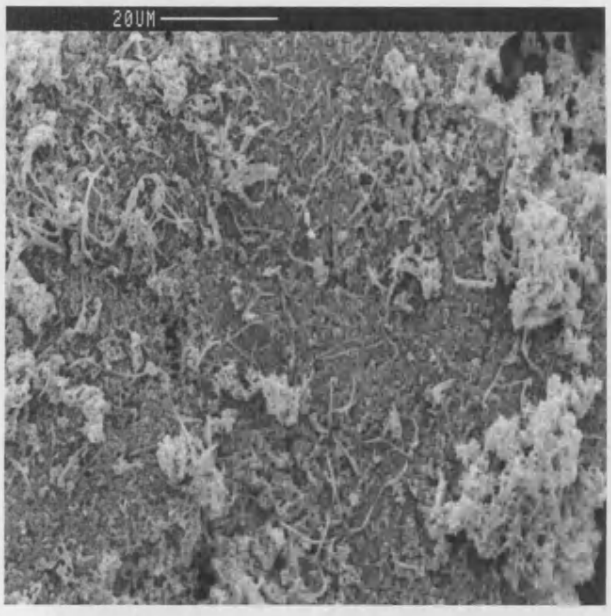


Figure 7.15 revealed that after 7 days (168 h) growth within the fermentor, the bulk of the substratum surface appeared to be covered in a heterogeneous microbiota. Figure 7.16 shows the biofilm was extremely dense and more diverse. Particularly evident were the filamentous cells, these were not evident at the earlier sampling points.

Figure 7.16: A SEM of a 168 h biofilm

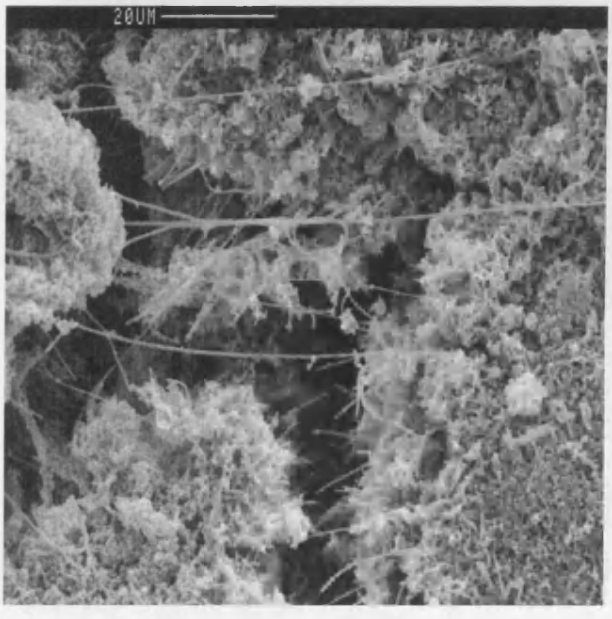


Figure 7.17: A SEM of a 240 h biofilm

Corn-cob structures were present within figure 7.17, these are formed via the close interaction between coccoid cells and filamentous cells. Figure 7.18 was a lower power electron micrograph of a biofilm. Whilst the biofilm did not cover the surface entirely, large aggregates seemed to cover the majority of the substratum after 240 h of biofilm formation and growth.

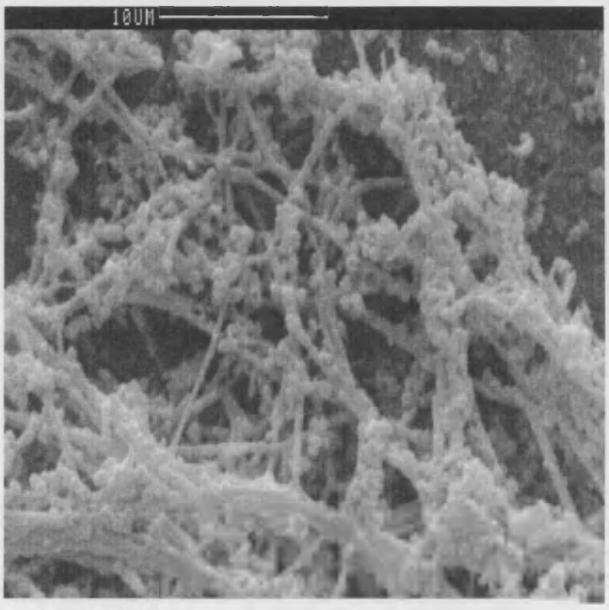


Figure 7.18: A SEM of a 240 h biofilm

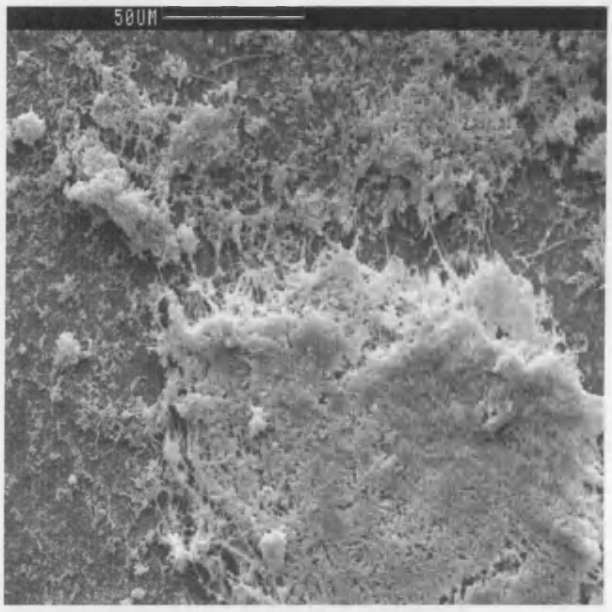
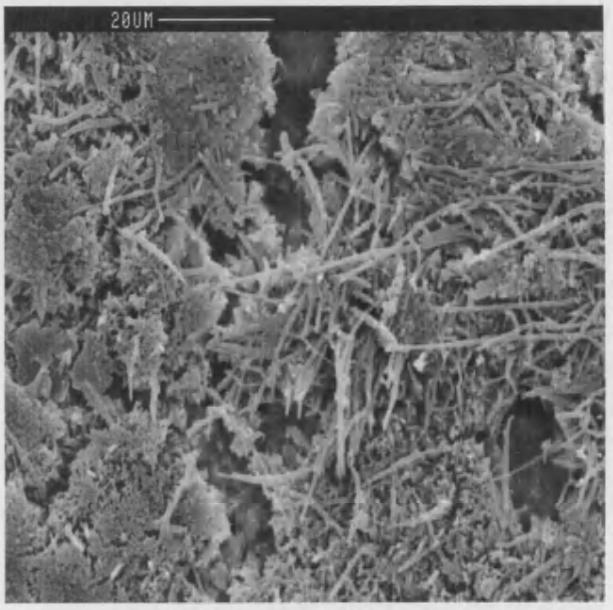
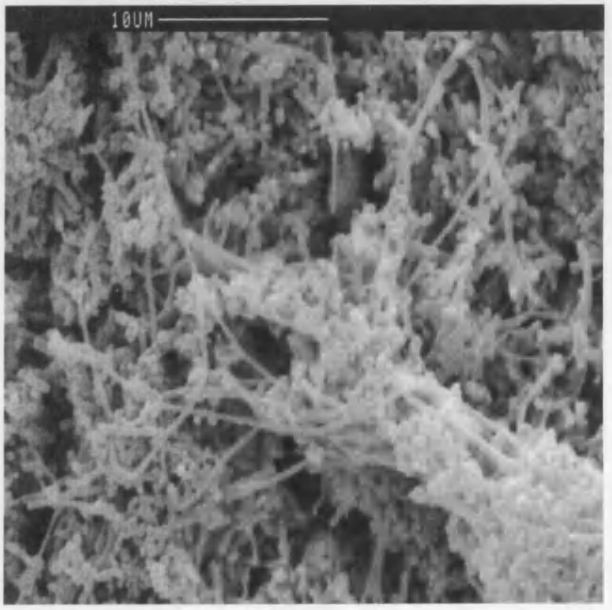


Figure 7.19: A SEM of a 336 h biofilm



Within Figure 7.19 the heterogeneity contained in the *in vitro* cultivated plaque is clear; a rosette arrangement formed by bacilli was present in the lower right hand corner. Figure 7.20 aptly demonstrated the diversity that was present within the CDFF, the differing morphotypes were in very close proximity with cocci and fusiforms.

Figure 7.20: A SEM of a 336 h biofilm



7.3.3 Transmission electron micrographs showing biofilm development

The data acquired via TEM complemented that garnered using SEM, allowing the differing morphotypes present within the biofilm to be visualised. In addition, TEM provided information as to the density of the (dehydrated) biofilm, whether morphotype stratification was evident, and how this varied as a function of biofilm age. No TEM's were produced of the 24 h biofilms as they proved to be of insufficient thickness for sectioning.

Figure 7.21: A TEM of a 72 h biofilm, magnification x 8 000

Figure 7.21 showed a dense biofilm of mainly coccoid cells, a ghost cell is apparent in the upper left hand corner of the micrograph.

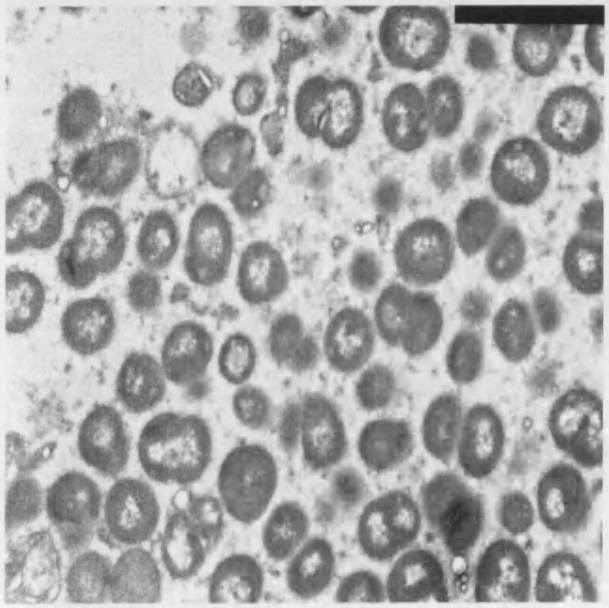


Figure 7.22: A TEM of a 168 h biofilm, magnification x 4 000

Figure 7.22 showed the various morphotypes of cells juxtaposed proximal to the biofilm surface. Long fusiform cells were distinguishable in the centre of the micrograph, there was an increase in the variety of morphotypes compared with what was seen after 72 h of cultivation.

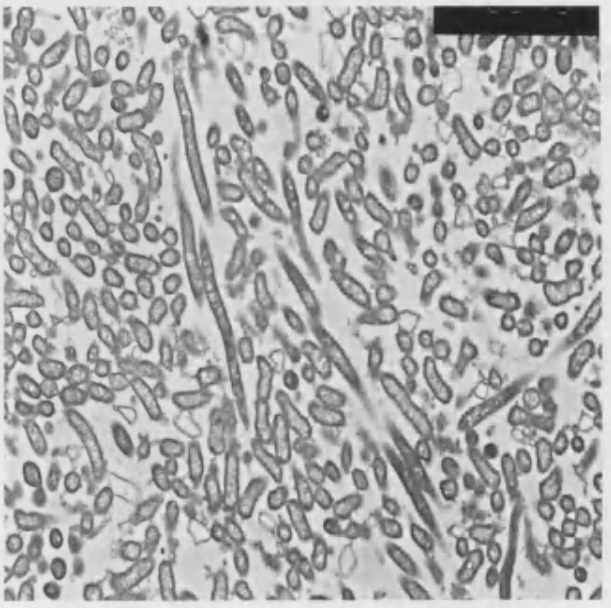


Figure 7.23: A TEM of a 168 h biofilm, magnification x 8 000

The biofilm surface was visible in figure 7.23. Rods were evident on the surface of the biofilm, of note were the cells which were encased within the denser (darker) matrix.

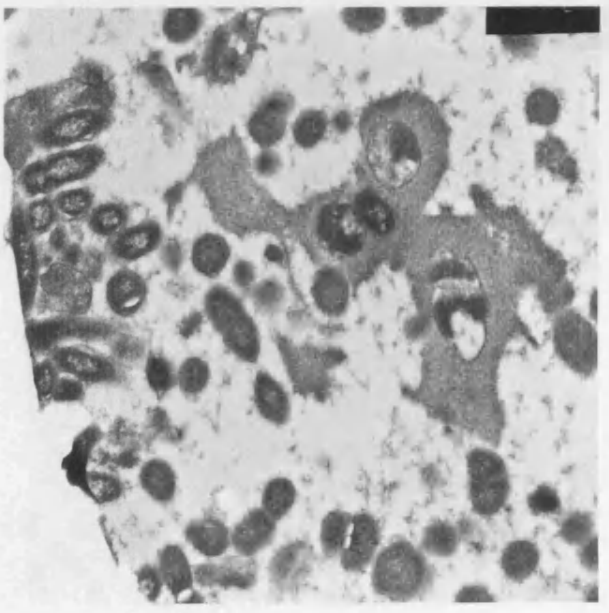


Figure 7.24: A TEM of a 240 h biofilm, magnification x 6 700

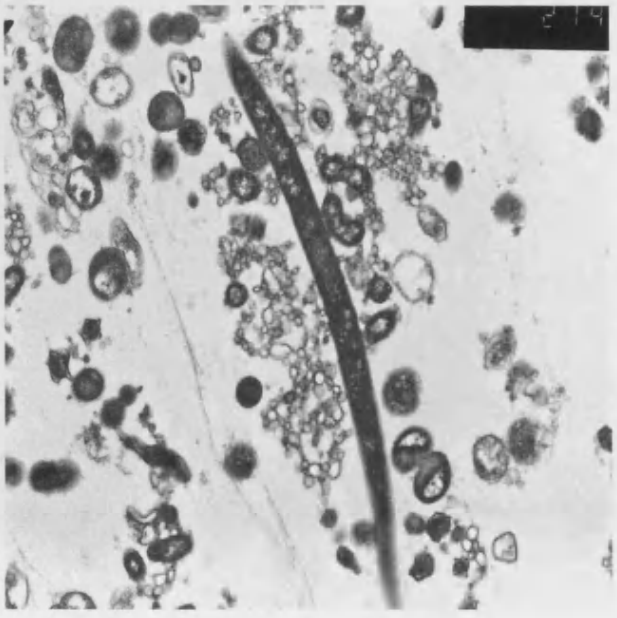


Figure 7.24 was dominated by a fusiform cell in the very centre of the micrograph, on closer examination the cells appeared to be co-aggregating, many different morphotypes of cells seemed to be interacting with the fusiform cell, possibly indicating many different co-aggregating events.

Figure 7.25: A TEM of a 240 h biofilm, magnification x 270

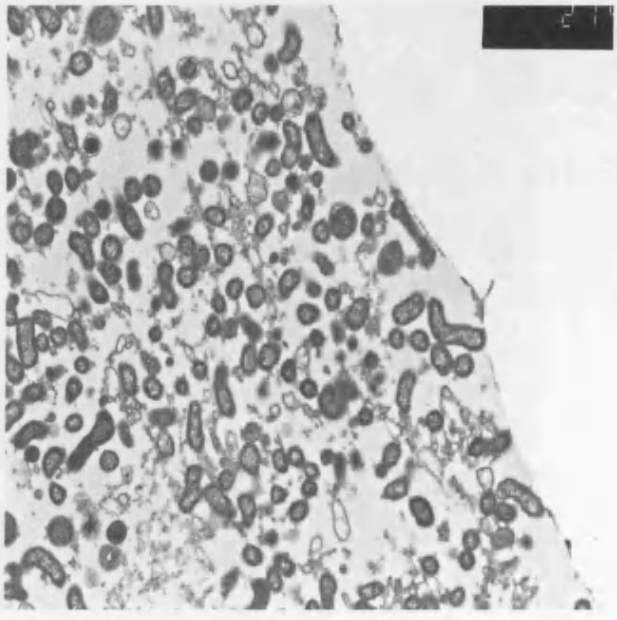
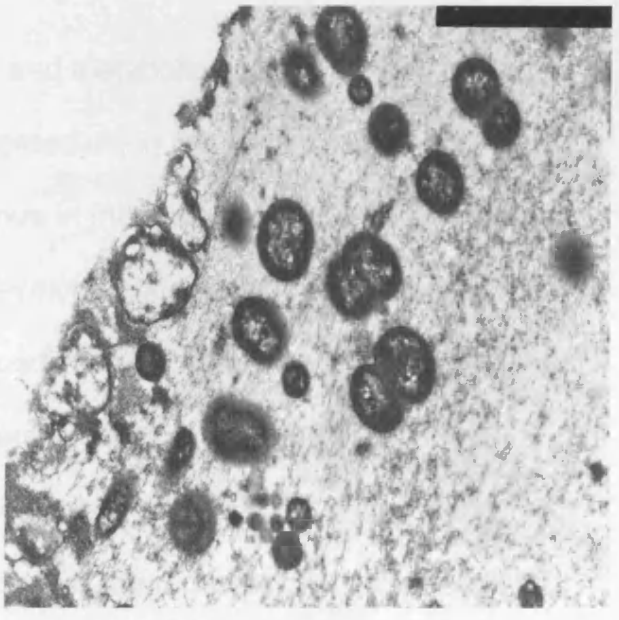


Figure 7.25 showed a thin section of a 240 h biofilm, the whole biofilm was visible within the micrograph. The biofilm substratum interface was present in the top right corner of the micrograph.

Figure 7.26: A TEM of a 336 h biofilm, magnification x 20 000

Figure 7.26 was a high magnification micrograph of a cluster of cells proximal to the biofilm surface interface, there they may have been co-aggregating, with interactions between the bacilli and the surrounding coccoid cells.

Figure 7.27: A TEM of a 336 h biofilm, magnification x 8 000



Within figure 7.27 the biofilm surface interface was visible in the top left hand corner. At the surface interface there are what appeared to be 'ghost' or dead cells evident, whereas further into the biofilm matrix the cells appeared to be healthy.

Figure 7.28: A TEM of a 336 h biofilm, magnification x 5 000

Both the biofilm/surface interface and the biofilm/substratum interface are visible in figure 7.28. Some fusiform/bacilli appeared to be present on the uppermost surface of the biofilm parallel to the biofilm/surface interface, in a protective arrangement of the cocoid cells deeper within the biofilm.

7.4 Discussion

In the previous chapter, the composition and metabolic plasticity of *in vitro* cultured microcosm plaques was investigated using the community analysis techniques, CLPP and DGGE. To continue in that vein, contained within this chapter is a visual reference of biofilm development over time within the CDFF. To optimise this approach, CLSM was used in conjunction with SEM and TEM to provide the maximal amount of optical detail possible regarding biofilm development over time. Electron microscopy is a powerful tool allowing the visualisation of the cells which form the biofilm, the morphotype of the cells and the way in which they are juxtaposed may be revealed. However due to the

method of sample preparation it is known that electron microscopy leads to the production of artefacts, which will not be present within pristine hydrated biofilms. Electron microscopy requires that the samples be dehydrated, hence the three-dimensional structure associated with mature biofilms will be masked, leaving an atypical condensed homogenous biofilm structure in its place. The use of CLSM provides some advantages over traditional electron microscopy and its inherent sample perturbation, allowing the visualisation of biofilm structure in real time coupled to nominal preparation of the sample (Watson., 1991). However, whilst the macroscopic features of biofilm structure are better represented by CSLM analysis, it does not provide the resolution necessary to visualise individual cells, or cellular morphotypes, which may be revealed via electron microscopy. Hence, in order to compile a comprehensive visual reference of biofilm development, a combination of techniques was utilised.

After 24 h of growth within the CDFF, the organisms present were rather sparsely distributed on the hydroxylapatite surface, as revealed by both CLSM and SEM. The CLSM analysis revealed that a rather high proportion of the organisms adhering to the substratum appeared to be non-viable, this concurred with other studies which have demonstrated that young and sparse dental plaque contained more non-viable than viable microorganisms (Netuschil et al.,1998). Certainly as has been suggested previously, dead cellular material seemed to compose an appreciable component of total plaque mass during initial plaque development (Brecx et al., 1981). Indeed the vitality of early plaque has been empirically

determined and was shown to be as low as 5 %, this increased significantly after 72 h to between 80 – 100% (Weiger et al., 1997). In addition, the young biofilms were low in morphotypic diversity, as the cells observable all appeared to be coccoid, again this agreed with *in vitro* electron microscopy of developing dental plaque which showed that coccoid cells predominate in early plaque formation (Listgarten et al., 1975, Nyvad et al., 1987b, Nyvad et al., 1987c, Takeuchi et al.,2001). However, in addition to the preponderance of coccoid cells that have been reported as principally composing dental plaque after 24 h, the presence of small numbers of both rod-shaped bacteria and filamentous bacteria has also been reported (Nyvad et al., 1987b), within the CDFF after 24 h of growth, coccoid cells were the sole morphotype visible. Indeed after 24 h of growth within the CDFF, the biofilms had not accumulated to a sufficient depth to permit TEM analysis, in contrast with plaque formation in the oral cavity (Listgarten, 1976, Nyvad et al., 1987c, Takeuchi et al., 2001). Perhaps the apparent hindered rate of initial biofilm formation within the CDFF may have been related to the way in which it was operated, the CDFF was inoculated for an 8 h period after which sterile medium was added to the system for the remainder of the run. The addition of sterile medium immediately after inoculation of the fermentor may have resulted in a dilution effect of the bacterial composition of the nascent biofilms, leading to the retardation of initial biofilm development within the CDFF.

After 72 h of biofilm growth within the fermentor, qualitatively there appeared to have been an increase in biofilm density. The confocal micrographs showed an

increase in the ratio of viable to non-viable cells, certainly compared to the confocal micrographs taken of 24 h biofilms. Whilst biofilm density increased, the electron micrographs did not show any significant expansion in morphotype diversity present within the biofilms, although chains of coccoid cells were evident that had not been apparent after 24 h growth, cocci were still the solitary morphotype present. This situation had changed after 168 h of growth within the fermentor. Confocal microscopy revealed that the biofilms were now denser than at either of the previous two sampling points, the biofilms were now dominated by viable cells. The scanning electron microscopy revealed that the substratum seemed to be covered in a heterogeneous microbiota, this was confirmed by the transmission electron micrographs, and several different morphotypes were evident including rod-like and fusiform cells. It has been previously reported that the major secondary colonisers of plaque are both Gram-positive coccoid and Gram-negative filamentous cells, whilst plaque maturation is often marked by a shift from Gram-positive coccoid to bacillary and pleiomorphic Gram-positive cells (Takeuchi et al., 2001). Indeed the pathogenicity of plaque is associated with a proliferation of Gram-negative rods and a shift from predominantly Gram-positive to predominantly Gram-negative organisms (Van Palenstein Helderman., 1981). Certainly, after 168 h of growth, the biofilms were now distinct from those which had been observed previously, with a high degree of diversification revealed by both the scanning and transmission electron microscopy.

The confocal micrographs of the 240 h biofilms revealed large numbers of both viable and non-viable cells, and structural motifs such as the distinctive microcolony formations which are synonymous with the biofilm lifestyle (Fig 7.8) (Costerton., 1995) were visible indicating that the biofilms were structurally mature. The scanning electron microscopy showed a profusion of corn-cob associations within the biofilms, these close associations have also been observed within dental plaque (Jones., 1972, Listgarten et al., 1973) although initially it was hypothesised that the corn-cob formations may actually represent a 'germinative phase of some filamentous forms', this idea had stretched back to the turn of the century where Viscentini in 1890 and 1897 described this corn-cob arrangement as a 'fruitful head', believing that the formation represented the progenitior stage of all dental plaque (Jones.,1972). It was eventually realised that this formation actually represented the close interaction of two distinct bacterial species (Listgarten et al., 1973). The transmission electron micrographs revealed a plethora of bacterial morphotypes that were present throughout the biofilm. Indeed one transmission electron micrograph showed the interaction of a fusiform with a number of differing cell types via microbial appendages, perhaps indicating the occurrence of co-aggregation events. Indeed dental plaque formation is dependent upon both intrageneric and intergeneric co-aggregation events. The central role performed by the fusiform bacterium *Fusobacterium nucleatum* in plaque maturation is known. The bacterium acts as a co-aggregation bridge between the early and late colonising bacteria, facilitating the development of the inherent heterogeneous nature of

dental plaque (Kolenbrander et al.,1993, Rickard et al., 2003), the transmission electron micrographs showed a fusiform cell apparently performing a very similar function within the *in vitro* grown bacterial biofilms of the present study. Physical interaction between differing species is crucial to biofilm formation. Biofilms formed from two plaque bacteria *Streptococcus cristatus* and *Porphyromonas gingivalis* within an *in vitro* model community demonstrated how physical interaction between two species resulted in the modulation of the pathogenic potential of the periodontopathogen *Porphyromonas gingivalis.* A *S. cristatus* surface protein was shown to repress the *P. gingivalis* fimbrial gene (*fimA*), hindering biofilm formation with *S. cristatus* (Xie et al., 2000). This exemplifies the complicated relationships, and delicate equilibrium which exists within dental plaque.

Confocal microscopy of the 336 h biofilms revealed a qualitative increase in the ratio of non-viable to viable cells compared to the 240 h biofilms, in addition, the biofilms analysed did not appear to be as dense as those observed after 240 h of growth. It has been reported previously that biofilms generated within the oral cavity and then visualised *ex-vivo* via CLSM produced mature differentiated biofilms, with the structural motifs associated with biofilm formation (Wood et al., 2000), the descriptions of those biofilms are consistent with the biofilms generated within the CDFF. However the biofilms described by Wood et al., 2000 were cultivated for 96 h before being examined, the relative cellular viability of the dental plaque as a function of time was not reported. Hence it is not known whether the observed qualitative increase in the ratio of viable to non-

viable cells to approximately the 168h sampling point, and the ensuing qualitative decrease in the ratio of viable to non-viable cells as the biofilms matured further (240/336 h biofilms), resulted from *in vitro* cultivation of the plaque, or whether this is a trend that is also evident in plaque *in vivo*. Indeed CLSM is now being employed in combination with fluorescence *in situ* hybridization (FISH) using species-specific oligonucleotides allowing detailed analysis of the architecture of biofilms with single cell resolution (Wecke et al., 2000). The transmission electron micrographs revealed how diverse the biofilms had become after 2 weeks of cultivation within the fermentor. Perhaps the apparent decreased level of viability was symptomatic of increased diversity, as the biofilm matured successional events resulted in biofilm diversification, however as biofilm age increased so inevitably did the number of nonviable cells trapped within the biofilm matrix, a number of ghost cells were apparent in a transmission electron micrograph. One transmission electron micrograph was particularly intriguing (Fig 7.28) showing fusiform cells parallel to the biofilm substratum interface on the surface of the biofilm in a protective arrangement of the cells deeper within the biofilm, although precise interactions were difficult to interpret without knowing precisely the micro-organisms involved. The diagnostic power of electron microscopy may be increased via the use of immunocytochemistry (Barber.,1995) , allowing the identification of cells composing the biofilm via immunolabelling, increasing what is known about interactions which occur within biofilms.

The results of this investigation have provided a visual reference as to biofilm formation within the CDFF over time. Initially the biofilms that were formed were homogeneous with a percentage of the microbiota adhering to the substratum being non-viable. As the biofilm developed, so diversity increased, as did biofilm density, as well as an apparent increase in the ratio of viable to non-viable cells, thereafter the biofilm continued to develop with fusiform cells particularly prevalent. However it did appear that at the final sampling point, after 2 weeks growth within the fermentor, the number of non-viable cells relative to viable cells was increasing, however this did not appear to have an impact on diversity, which if anything appeared to continue to increase. The biofilms which were generated within the fermentor mimic the reported development of dental plaque *in vivo,* with many of the reported structures and motifs incorporated into the biofilms cultivated within the CDFF. The results reported in this chapter form a visual reference to accompany the metabolic and phylotypic analysis of the biofilm community, reported in the previous chapter. In the next chapter, the bacterial composition of the biofilms was analysed over time using both traditional and PCR-based techniques, complementing the community-based analysis reported in this and the previous chapter.

CHAPTER EIGHT

CHARACTERISATION OF CDFF CULTIVATED SUB-GINGIVAL BIOFILMS

8.1 Introduction

The previous two chapters of this investigation have centred upon the analysis of the complete biofilm community and how this evolved as the biofilm developed. This chapter is concerned with the characterisation of that community and the diverse bacterial species present within the biofilm. To that end, two different techniques were employed, both of which have previously been widely used in the analysis of biofilm composition: (i) viable counts, utilising both selective and non-selective agar (Kamma et al., 2000) and (ii) molecular characterisation of the cultivable microbiota. The use of viable counts as a method of quantifying biofilm composition is a classical bacteriological technique, whereas molecular characterisation is reliant upon PCR to amplify the 16S rRNA genes, the subsequent sequencing (and analysis) is used to detect variations within the 16S rRNA of the different biofilm bacteria. PCR has previously been harnessed to determine species diversity within subgingival plaque with particular reference to the large number of unculturable bacteria *in vivo*, these have been acknowledged as constituting a significant portion of the microbiota within the oral cavity (Paster et al., 2001, Sakamoto et al., 2000). Indeed, it has previously been estimated that only 50 % of the microbiota of the oral cavity may be cultivable (Socransky et al., 1963). The molecular identification of the cultivable bacteria from the CDFF presents an opportunity to compare with what has previously been identified from within periodontal pockets using both traditional and molecular methodologies. Additionally, the use of these techniques allowed both the gross compositional changes and quantification of the individual bacteria present at each particular

sampling point within the biofilm to be monitored over time, viable counts are a rapid way to gauge the approximate biofilm composition, however, sequencing allowed the precise identification of the cultivable microbiota. Hence, as the biofilm developed within the CDFF any fluctuations in the microbiota comprising the biofilm were monitored.

8.2 Materials and Methods

The CDFF was set up, inoculated and sampled as described in chapter 6, section 6.2

8.2.1 Bacteriological characterisation of CDFF-cultivated biofilms

The method used was the same as that described in chapter 2, section 2.3, with the following exceptions:

1. Two pans (10 biofilms) were removed aseptically from the CDFF. The discs (plus their associated biofilms) were placed in 30 ml of PBS and vortex-mixed for a period of 2 minutes which allowed for full biofilm removal from the discs. 28.5 ml of the suspension was required for the CLPP analysis, described in chapter 6, the remainder was utilised for bacteriological characterisation using selective and non-selective media.

8.2.2 Molecular identification of cultivable bacteria

8.2.2.1 Sub-culturing of distinct colony morphotypes

After incubation aerobically for 24 h, in the case of CBA plates, or for up to 7 days for FAA plates incubated within an anaerobic cabinet, the plates were removed and the distinct colony types on the agar plates were counted. Once the number of each distinct colony type had been recorded, each colony type was sub-cultured onto the appropriate non-selective agar and incubated either aerobically or anaerobically, dependent upon the plate the colony had originally been isolated from. Sub-cultures were subjected to a preliminary categorisation via Gram reaction, catalase and oxidase tests. The sub-cultures were then catalogued and frozen in BHI broth (Oxoid Ltd) with 10% glycerol (Sigma Ltd) and stored at - 70° C.

8.2.2.2 PCR amplification of the 16S gene

Identification to species level of the sub-cultured bacteria was carried out by PCR amplification and partial DNA sequencing of the 16S rRNA gene. Prior to PCR amplification, the sub-cultured bacteria were revived on the appropriate agar medium. PCR amplification was performed with a Primus 25 Thermocycler (MWG Biotech, Milton Keynes, U.K) using 10x NH_4-based reaction buffer (Bioline, London, U.K) containing 10 mM $MgCl_2$, 2 mM of deoxynucleotide triphosphates, 0.9 pmol of each primer, 1.5 U *Taq* DNA polymerase (Bioline U.K) and 1 individual colony taken from the appropriate agar plate, this was in a total volume of 100 µl. The 16S rRNA gene conserved primers used for this first

round PCR were 27F and 1492R (See chapter 6, table 6.1) (Genosys Biotechnologies Ltd, Cambridgeshire, U.K). The samples were then amplified as follows: 94 ℃ for 5 min, followed by 29 cycles of 94 ℃ for 1 min, 54 ℃ for 1 min and 72 ℃ for 1.5 min, with a final extension at 72 ℃ for 5 min. The presence of product was confirmed on a 1 % agarose gel (Amresco, NBS Biologicals, Huntingdon, U.K). The PCR products were then purified using a commercially available DNA purification kit (Qiagen, Crawley, U.K), producing 30 µl of purified product per sample.

8.2.2.3 Sequencing reaction

The sequencing reaction was performed with a Primus 25 Thermocycler (MWG). Each reaction mixture contained 1 µl of cleaned product, 0.7 pmol of the16S rRNA gene conserved primer 357F ([5'- CTCCTACGGGAGGCAGCAG – '3] Genosys Biotechnologies Ltd), 2 µl of Big dye Mix (ABI, Warrington, U.K) with water added to obtain a final volume of 7 µl. The samples were then amplified as follows: 95°C for 5 min followed by 99 cycles of 95°C for 30 s, 55°C for 20 s, 65°C for 4 min.

8.2.2.4 Clean-up of the sequencing reaction

On completion of the sequencing reaction, ethanol precipitation of the DNA was performed, to each reaction was added 15 µl of sdH_20, 2 µl of 3M sodium acetate (BDH) and 50 µl of 95 % ethanol, chilled to -20℃ (BDH), this was then incubated on ice for 20 min. The mixture was then centrifuged at 16,000 g for 25 min at

4°C, the liquid phase was then removed. Whereupon 250 µl of 70 % ethanol, chilled to -20°C, was added, this was then centrifuged at 16,000 g for 15 min at 4°C and the liquid phase was then removed. The precipitated DNA was placed on a hot-block set at 95 °C for 20 s, to remove any residual ethanol. The DNA pellet was then re-suspended in 20 µl of Template Supressor Reagent (ABI). The samples were analysed on an ABI PRISM 310 Genetic Analyser. Identifications were obtained by comparing the sequence data generated (c.350-500 bases) with the database at both the Ribosomal Database Project (RDP) at Michigan State University (Maidak et al., 2000, http://rdp.cme.msu.edu) and BLAST at the National Centre for Biotechnological Information (Altscul et al., 1997, http://www.ncbi.nlm.nih.gov/BLAST).

8.3 Results

8.3.1 Bacteriological composition of CDFF-cultivated biofilms

The CDFF was set-up and run in an identical manner on two separate occasions. In this chapter, the viable count data and sequencing of the cultivable flora are presented.

Figure 8.1: Viable counts from run 1 showing the composition of the sub-gingivally derived biofilms grown within the CDFF as a function of time.

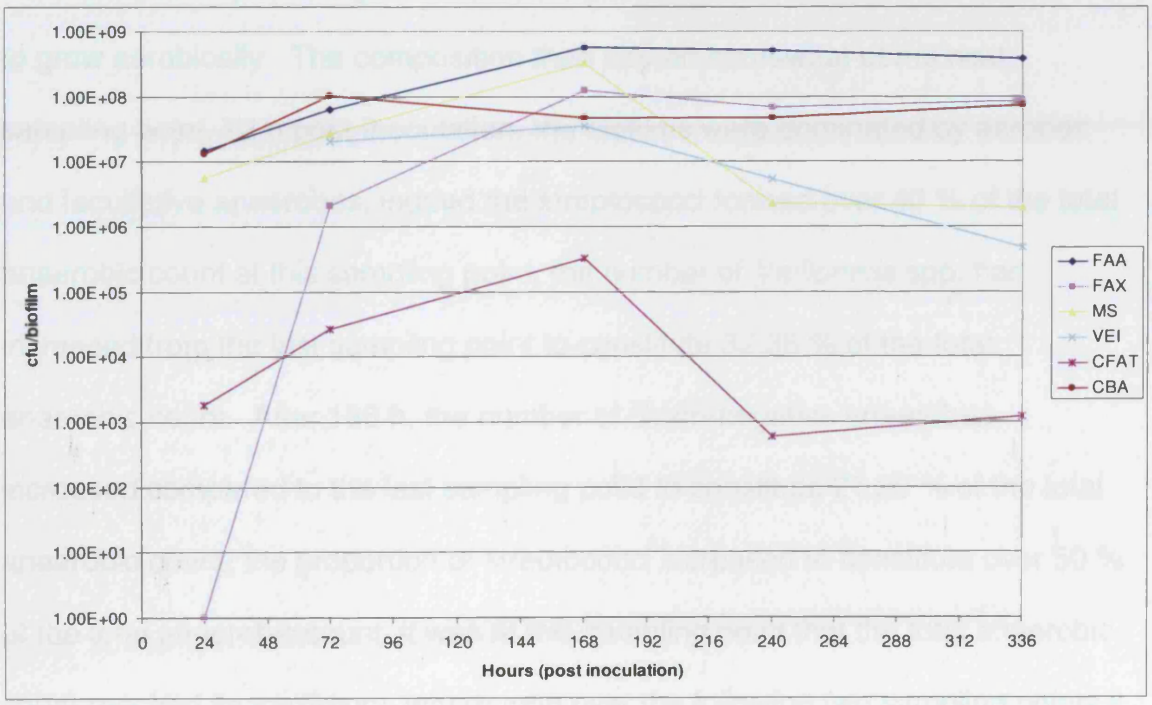


Table 8.1: The composition of the biofilms from run 1 relative to the total anaerobic count

	% of total anaerobic count				
	24 h	72 h	168 h	240 h	336 h
Gram-negative anaerobes	0.00	3.33	21.29	13.57	23.06
Streptococci	37.86	41.90	56.64	0.45	0.53
Veillonela spp.	0.00	32.48	6.08	1.06	0.13
Actinomyces spp.	0.01	0.04	0.06	0.00	0.00
Aerobes/facultative anaerobes	90.53	160.95	8.08	9.17	20.00

At the first sampling point after 24 h of growth within the fermentor, the biofilms were composed principally of streptococci and a variety of microorganisms able to grow aerobically. The composition then altered somewhat at the next sampling point, 72 h post-inoculation, the biofilms were dominated by aerobes and facultative anaerobes, indeed the streptococci formed over 40 % of the total anaerobic count at this sampling point, the number of *Veillonella* spp. had increased from the last sampling point to constitute 32.38 % of the total anaerobic count. After 168 h, the number of Gram-negative anaerobes increased compared to the last sampling point to constitute 21.29 % of the total anaerobic count, the proportion of streptococci increased to constitute over 50 % of the total anaerobic count, it was at this sampling point that the total anaerobic count reached its maximum, whereupon over the following two sampling points it decreased. At the 240 h sampling point, the number of *Veillonella* spp. constituting the total anaerobic count had decreased from its maximum after 72 h to constitute 1.06 % of the total anaerobic count. The *Actinommyces* spp. which were no more than minor constituents of the biofilm at the previous sampling points, decreased in numbers at the 240 h sample point to hover just above

detectable limits. After 336 h, the Gram-negatives now formed the largest group present within the biofilms, both the *Veillonella* spp. and the streptococci formed less then 1 % as a proportion of the total anaerobic count, however the number of aerobes/facultative anaerobes had increased in numbers at both the 240 h and 336 h sampling point.

Figure 8.2: Viable counts from run 2 showing the composition of the sub-gingivally derived biofilms grown within the CDFF as a function of time.

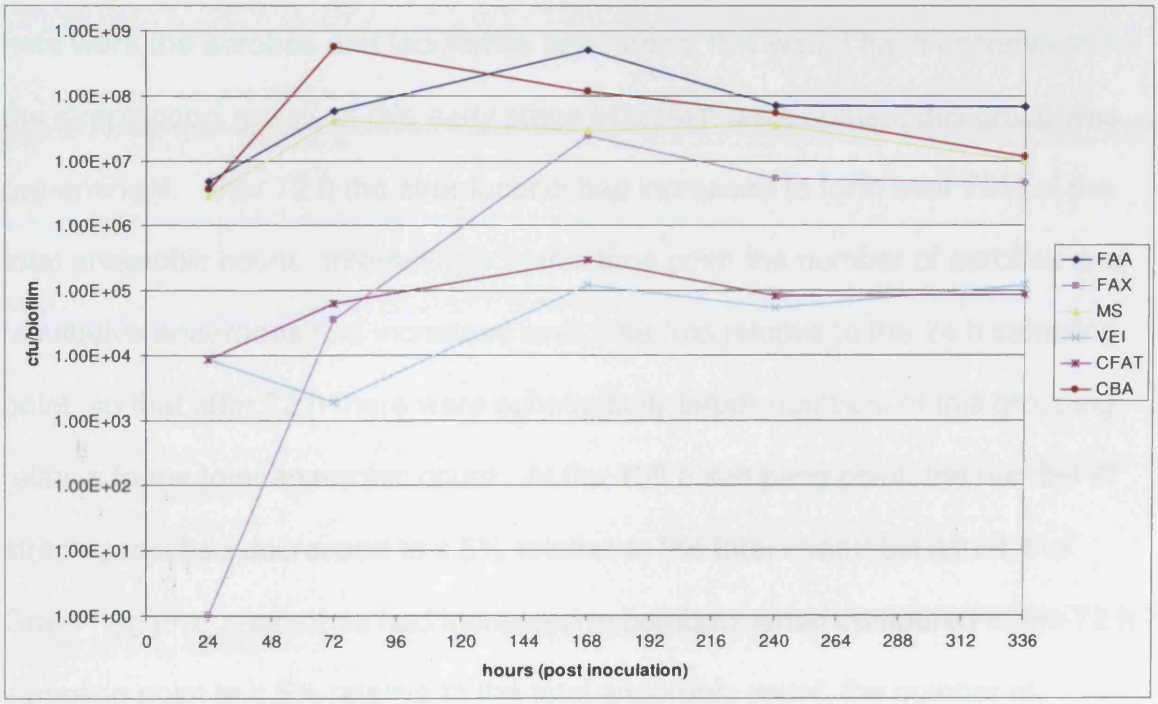


Table 8.2: The composition of the biofilms from run 2 relative to the total anaerobic count

	% of total anaerobic count				
	24 h	72 h	168 h	240 h	336 h
Gram-negative anaerobes	0.00	0.06	4.76	7.69	6.94
Streptococci	60.26	71.13	5.85	49.57	14.86
Veillonela spp.	0.18	0.00	0.02	0.08	0.18
Actinomyces spp.	0.18	0.11	0.06	0.12	0.13
Aerobes/facultative anaerobes	78.21	948.45	23.90	76.07	17.30

After 24 h of growth within the fermentor, the predominant bacterial group was the streptococci which constituted over 60% of the total anaerobic count, also of note were the aerobes and facultative anaerobes, this would have encompassed the streptococci group, at this early stage of biofilm development this group was pre-eminent. After 72 h the streptococci had increased to form over 70% of the total anaerobic count. Interestingly at this time point the number of aerobes and facultative anaerobes had increased over nine-fold relative to the 24 h sampling point, so that after 72 h there were substantially larger numbers of this grouping relative to the total anaerobic count. At the 168 h sampling point, the number of streptococci had decreased to c.5% relative to the total anaerobic count, the Gram-negative anaerobes had increased in numbers when compared to the 72 h sampling point to c.5% relative to the total anaerobic count, the number of aerobes/facultatively anaerobic bacteria had decreased relative to the total anaerobic count such that there were c.20% of the total anaerobic count after 168 h. After 168 h growth within the CDFF, the total anaerobic count reached its zenith, at the following two sampling points the bacterial counts progressively decreased. After 240 h the number of streptococci had increased as a proportion

of the total anaerobic count to constitute c.50% of the cultivable microbiota, this increase was repeated with the aerobes/facultatively anaerobic grouping which had increased to c.75 % relative to the total anaerobic count. At the 336 h sampling point, the streptococci and aerobe/ facultatively anaerobic count decreased as a proportion of the total anaerobic count. At all sampling points, the *Veillonella* spp. and the *Actinomyces* spp. constituted c.<1% as a proportion of the total anaerobic count.

There are broad similarities in how the biofilms developed during both runs as revealed by viable counts. During both runs the total anaerobic count increased during the first three sampling points (24 h, 72 h, and 168 h). During both runs the total anaerobic count reached its maximal after 168 h of growth within the fermentor. At the final two sampling points (240 h and 336 h) the total anaerobic count then progressively decreased, although this reduction in the total anaerobic count was perhaps more pronounced during run 2. Indeed, during both runs the aerobic / facultatively anaerobic bacteria initially proliferated rapidly with the Gram-negative anaerobic grouping increasing as a proportion of the total anaerobic count as the biofilm matured.

8.3.2 The cultivable bacteria present within the CDFF at each sample point as determined by partial sequencing of the 16S rRNA gene.

Table 8.3: The number of aerobe, facultative anaerobic and anaerobic taxa found at each time point during both run 1 and run 2.

	Run	Hours post-inoculation				
		24	72	168	240	336
aerobe	1	2	1	1	1	1
	2	4	1	2	1	2
anaerobe	1	1	3	4	4	3
	2	0	2	5	5	5
facultative	1	5	5	6	5	6
	2	2	6	8	6	4

Table 8.4: The Gram classification of the bacterial taxa found at each time point for both run 1 and 2.

	Run	Hours post-inoculation				
		24	72	168	240	336
Gram positive	1	7	7	8	7	5
	2	5	5	11	9	8
Gram negative	1	1	2	3	3	5
	2	1	4	4	3	3

Table 8.5: The cultivable bacteria detected at each sampling point during both run 1 and run 2 (the light blue bars denote taxa isolated during run 1, and the dark blue run 2).

Identified bacterium	Run	Hours post-inoculation				
		24	72	168	240	336
Actinomyces hyvoginalis	1					
	2					
Actinomyces meyeri	1					
	2					
Actinomyces naeslundii	1					
	2					
Actinomyces siumastidis	1					
	2					
Bulledia moorei	1					
	2					
Eikenella corrodens	1					
	2					
Eubacterium yurii	1					
	2					
Fusobacterium nucleatum	1					
	2					
Gemella moribillorum	1					
	2					
Granulicatella adiacens	1					
	2					
Haemophilus parainfluenzae	1					
	2					
Haemophilus segnis	1					
	2					
Kingella denitrificans	1					
	2					
Kocuria rhizophila	1					
	2					
Micrococcus luteus	1					
	2					
Micromonas micros	1					
	2					
Neisseria gonorrhoeae subgroup	1					
	2					
Porphyromonas gingivalis	1					
	2					
Propionibacterium acnes	1					
	2					
Rothia mucilaginosa	1					
	2					
Staphylococcus epidermidis	1					
	2					
Staphylococcus haemolyticus	1					
	2					
Staphylococcus hominis	1					
	2					
Staphylococcus warneri	1					
	2					
Streptococcus anginosus	1					
	2					
Streptococcus pneumoniae subgroup	1					
	2					
Streptococcus sanguinis	1					
	2					
Streptococcus sinesis	1					
	2					
Veillonella dispar	1					
	2					
Virgibacillus proomii	1					
	2					

Table 8.3 categorises the cultivable taxa identified throughout the duration of both run 1 and run 2. 24 hours following the inoculation of the fermentor during run 1, of the eight different taxa isolated and identified, 5 were facultative anaerobes, 1 was an strict anaerobe with a further two species being aerobes (table 8.3). This composition had changed somewhat at the second sampling of the fermentor, 72 h post inoculation, the number of anaerobes increased somewhat as three different taxa were isolated, the number of facultatively anaerobic taxa remained the same and the number of aerobic taxa isolated was reduced to 1, which was to remain constant over the remainder of sampling points. At 168 h post-inoculation, the total number of different taxa isolated and identified was at its maximal, the number of anaerobes isolated increased relative to the 72 h sampling point to 4 as did the number of facultatively anaerobic taxa which increased in number to 6. At the final two sampling points the number of identified taxa remained fairly constant although significantly the predominant group at all sampling points was the facultative anaerobes. These subtle shifts in composition were reflected in the particular organisms identified at each time point. The streptococci, and the *Neisseria* spp. were ubiquitous, with representatives being isolated at all time points throughout run 1. Certainly, the prevalence of the streptococci and *Staphylococcus* spp. helped to explain the preponderance of facultative anaerobes isolated throughout the course of the run. Interestingly, fastidious anaerobes such as *P. gingivalis*, *F. nucleatum* and *Bulledia moorei* were only detected via culture at the 168 h time point. One must assume that these organisms were present within the confines of the biofilm but

only detectable via culture after a week of biofilm formation, when they had formed a large enough percentage of the total flora to be detectable.

During run 2, 24 h after inoculation of the fermentor a total of 4 aerobes were isolated and identified (table 8.3), no anaerobic organisms were detectable via culturing whilst 2 facultatively anaerobic taxa were identified. At the next sampling point (72 h post-inoculation of the fermentor), the composition of the identified taxa had changed considerably to now include one aerobe, whilst the number of anaerobic taxa had increased to 2,and the number of facultatively anaerobic isolates identified increased to 6. At 168 h post-inoculation, all groups had increased in number relative to the 72 h sampling point, with 8, 5 and 2 facultatively anaerobic, anaerobic and aerobic taxa respectively, totalling 15 different taxa identified. After 240 h, 12 different taxa were isolated and identified. At the final sampling point, the number of identified taxa decreased again compared to the 240 h sampling point, 2 aerobic isolates were identified along with 5 anaerobic isolates which now comprised the largest group, whilst the number of facultatively anaerobic isolates had decreased and numbered 4. The streptococci were isolated at all time points (table 8.5), contributing to the number of facultative anaerobes identified however, very few staphylococci were detected. *F. nucleatum* was isolated after 72 h along with other anaerobic species such as *V. dispar.* However, after 168 h the numbers of anaerobic taxa reached their zenith, with organisms such as *Eubacterium yurii* and *Bulledia moorei* identified as part of the microbiota. As was noted with run 1, although

these must have been present within the biofilm their numbers must have lingered below detectable levels until conditions were conducive to their proliferation.

Table 8.4 catalogues an interesting phenomenon that was observed during the course of both runs, at each sampling point the number of Gram-positive taxa identified was the same as or exceeded the number of Gram-negative taxa identified.

Table 8.5 allows a comparison to be made of the bacterial constituents of the biofilms throughout each respective run, in contrast to run 1 *P. gingivalis* was not isolated during run 2 but the aerobic bacterium *Virgibacillus proomi* was only identified at the 24 h sampling point during run 2 and was not detected at all during run 1. Indeed, during run 1, 24 h post-inoculation the facultative anaerobes constituted the greatest number of individual taxa which contrasts with the 24 h point during run 2, where the aerobes constituted the greatest number of individual isolates. Indeed, there were elevated numbers of individual aerobic species identified during run 2 when compared to run 1, especially at the 24 h sampling point. In total, there were only 3 taxa identified during run 1 that were not detected at any time point throughout run 2. Of these 1 was the anaerobe *P. gingivalis*, whilst the others were facultatively anaerobic, *A. siumastidis* and *S. haemolyticus*. However, 10 taxa were identified during run 2 which were not present at any time point during run 1, of these 7 were present

transiently, being present at a single sampling point and not detected again. Of the 10 taxa, 4 were aerobic (*K. denitrificans, K. rhizophila, M. luteus* and *V. proomi*), 3 were facultatively anaerobic (*H. segnis, S. anginosus* and *S. sinesis*) whilst 3 were anaerobes (*A. hyvoginalis, A.meyeri* and *E. yurii*).

Another disparity between runs 1 and 2 was the turnover rate of bacterial taxa, which was higher during run 2, than during run 1. During run 2, a larger number of novel isolates at each sampling point were identified that had not been seen previously during the run. After 72 h of growth, during run 1 there were 2 novel taxa identified, however at the corresponding sampling point during run 2, there were 6 novel taxa which had not been seen at the 24 h sampling point. Indeed even after 240 h of cultivation within the fermentor there were 6 taxa which had not been identified previously during run 2, conversely, the corresponding time point during run 1 produced no novel isolates.

Discussion

The results presented in this chapter represent the culmination of a polyphasic approach to the analysis of the biofilms generated within the CDFF over the course of 2 runs. Whereas the two preceding chapters concerned themselves with a community analysis, this chapter focused upon the documentation of the biofilm bacterial constituents. To that end, two differing yet complementary techniques were employed, the use of viable counts to gain an understanding of fluctuations within the main generic groups composing the biofilm and sequencing of the cultivable microbiota to provide increased resolution to ascertain the precise bacterial taxa present within the biofilm at each sampling point. Both of these techniques were reliant upon the cultivation of the microbiota, this being merely a preliminary step as regards sequencing of the bacteria.

The scrutiny of biofilm composition documented within this chapter was the combined analysis of two CDFF 'runs', each run being conducted over a 14 day period, with sampling at pre-determined times. Interestingly, the organisms isolated throughout the course of each run varied; although there were only three bacteria identified during run 1 that were not seen throughout the course of run 2, there were 10 bacteria identified throughout the course of run 2 that were not seen at any of the sampling points during run 1. This disparity results in a quandary, were these gross differences in biofilm composition a result of minute compositional differences in the inoculum, perhaps the compositional differences

may be due to the model itself whereby conditions may have differed somewhat between run 1 and run 2 or it may have been due to a failure to culture bacteria which may have been present, but below detectable levels. It is likely, however, that all of these factors played a role in the observed compositional differences between the two runs. Whilst strenuous efforts were made to replicate experimental conditions precisely, the complex nature of the study meant that all of the outlined experimental imponderables above probably contributed to the observed differences in bacterial composition between the two runs. Hence, due to the experimental vagaries outlined, a fuller understanding of the composition of microcosm plaques cultivated within the CDFF may be forthcoming if the summation of both runs is analysed, rather than the analysis of both runs as stand-alone experiments. Indeed, even the precise composition of periodontal pockets will vary from site to site within a single patient (Socransky., 1970), thus emphasising the complexity and polymicrobial nature of periodontal diseases. Moreover, focusing too intently upon differences in composition between runs risks becoming embroiled in the minutiae, both run 1 and run 2 progressed from a microbiota that was predominantly facultative/aerobic to one that came to include increasing numbers of anaerobic bacteria concomitant with biofilm development.

Bradshaw et al., (1996) investigated the effect of several experimental parameters on the development of defined mixed-culture biofilms. The effect of aeration upon 10 species, associated with both oral health and disease,

comprising aerobic, facultatively anaerobic and anaerobic bacteria was investigated. To this end, a two-stage chemostat system was employed, the first being anaerobic, the second aerobic. It was found that anaerobic species were able to both survive and multiply within the aerobic second stage, the majority of which were found to increase in number due to successional changes within the confines of biofilms. Moreover, the rate of addition of culture from the first to second stage was reduced to determine whether the numbers of anaerobic bacteria present in the second stage was due to bacterial growth. The data revealed that the anaerobes were indeed growing in the aerated culture, although numbers were concentrated within the confines of mature biofilms. These data correlate with what was found during the course of this study. Low levels of oxygen were introduced into the fermentor, but anaerobic bacteria were identified as being present within the mature biofilms; confirmation perhaps that the biofilm lifestyle provides microhabitats suitable for the growth of oxygen-intolerant bacteria, even when the surrounding environment is oxygenated. Perhaps the metabolic activity of some bacterial constituents altered local conditions within the biofilm distal to the biofilm-air interface, permitting the growth of oxygen-intolerant species. It is known that *F. nucleatum*, an anaerobic species which is nonetheless tolerant of aerated environments, facilitates the growth of the fastidious anaerobe *P. gingivalis* in both aerated and CO_2-depleted environments (Diaz et al., 2002). *F. nucleatum* was detected during both fermentor runs, interestingly however *F. nucleatum* was detected concomitantly with *P. gingivalis* at the same sampling point during run 1, *P. gingivalis* was

isolated despite the microaerophilic conditions within the fermentor providing circumstantial evidence of *F. nucleatum* aiding the growth of *P. gingivalis*. *P.gingivalis* was not detected at any sampling point during run 2. The failure to detect *P. gingivalis* throughout the course of run 2 is less surprising when one considers the disparity in numbers of Gram-negative anaerobic bacteria detected during run 2, compared to run 1. At the 168 h sampling point during run 1 Gram-negative anaerobes constituted 21.29 % of the total anaerobic count, this contrasts somewhat with the same sampling point during run 2 where Gram-negative anaerobes constituted a mere 4.76 % of the total anaerobic count. Hence, to not detect a fastidious anaerobe such as *P. gingivalis* during the course of run 2 was not an unexpected finding.

A trend that was apparent during both runs was the increase in viable counts over the first two sampling points. The increase in viable counts reached a peak at the 168 h sampling point whereupon it then slowly declined. This seemed surprising initially, but merely mimics what is found *in vivo*, where observed rates of microbial generation time at initial stages of plaque formation are considerably lower than those found within older plaques (Weiger et al., 1995).

The resolution provided via the use of viable counts may be increased by the use of selective agars which are formulated to encourage the growth of a specific genus or bacterial group to the preclusion of other groups that may be found as part of the oral microbiota. As a part of this study, both non-selective and

selective bacterial growth media were used; *Actinomyces* spp. were isolated on CFAT agar, *Veillonella* spp. on Veillonella agar, streptococci on MS agar and the isolation of Gram-negative anaerobic species with FAX that incorporated both vancomycin and nalidixic acid. This permitted the relative proportions of the major oral genera, at each sampling point, to be established. However, organisms such as *A. actinomycetemcomitans* and *Tannerella forsythensis* which have been positively correlated with periodontal diseases (Moore and Moore., 1994, Ximenez-Fyvie et al., 2000) were not detected at any sampling points during either of the two runs. This may have been due to a failure to cultivate organisms such as these within the CDFF. Alternatively, they may have been present within the biofilms, but failed to grow on any of the media selected for use during the study, or they may not have been present within the inoculum. Selective media has been described previously for the cultivation of *Bacteriodes* spp, *A. actinomycetemcomitans* and other putative periodontal pathogens (Slots., 1986). However one must bear in mind the efficacy of culturing as a method for detection of a particular bacterium within a microhabitat. A study by Loesche et al., (1992) concluded that DNA probes and immunological reagents were superior to the culture approach for the detection of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythus*. Therefore the use of a wider range of selective media was not considered to be prudent, given the unlikely increase in resolution afforded by their use over and above that provided by the selective media that were used as part of this study. Recently, a methodology has been described using a simulated natural environment, a diffusion chamber, to isolate

in pure culture previously 'uncultivable' marine bacteria (Kaeberlein et al., 2002). If such approaches could be adapted, they would prove useful in the modeling of the periodontal pocket and the cultivation of bacteria which are known to be present *in vivo* but often prove rather difficult to culture *in vitro*. Although one must acknowledge the microbiota and chemical components composing a marine environment are more readily available and perhaps easier to model, than the biotic components and allied physiological conditions prevailing within a human periodontal pocket.

An objective at the outset of this study was to catalogue the different microbial taxa isolated from a CDFF which had been seeded with an inoculum consisting of homogenised sub-gingival plaque. Gram-positive taxa were the predominant isolates at all sampling points during both fermentor runs (table 8.4). Gingival inflammation is considered to be associated with elevated numbers of Gram-negative rather than Gram-positive species (Moore and Moore., 1994). Does the apparent abundance of Gram-positive taxa isolated demarcate a bias or skew favouring the growth of Gram-positive bacteria inherent within the model, or culture media used, or could it represent an accurate maturation of the microbiota within the inoculum?. A study carried out to examine the bacteria present in subjects with adult periodontitis (Ximenez-Fyvie et al., 2000) commented on the frequency with which the Gram-positive *Actinomyces* species were isolated both in supra and subgingival plaque. Bacteria isolated during the course of this study; *S. sanguinis, A. naeslundii, P. acnes, S. heamolyticus* and

V. dispar were more frequently found in supra rather that subgingival sites in studies examining the microbiota associated with periodontitis in adults (Moore at al., 1982, 1983). Addiitionally; *F. nucleatum, M. micros, A. meyeri, A. naeslundii, P. gingivalis* and *S. epidermidis* were isolated by Moore et al subgingivally more frequently than they were isolated supragingivally, these bacteria were also isolated during the course of this study. Both *A. naeslundii* and *F. nucleatum* are the most commonly occurring species in the human gingival crevice, whilst *F. nucleatum* is the most frequently identified pathogen isolated from periodontal lesions (Moore and Moore., 1994)-both of these bacteria were identified during the course of both runs in this study. However, Tanner et al., (1994) reported that *M. micros* is the most frequently isolated species from human subgingival plaque, this species was also identified as a bacterium present in this study. In another study by Tanner et al., 1998 examining patients with initial periodontal lesions; *F. nucleatum, S. anginosus, P. ginivalis, E. corrodens, A. gerencseriae, M. micros, G. morbillorum* and *S. sanguinis* were isolated amongst others, these were also isolated during the course of this study. This may lead one to conclude that the consortia identified as having being present at each sampling point was a representative selection of bacterial species and not wholly atypical of what one could reasonably expect to find within a periodontal lesion. Although the bacteria isolated do not represent an exhaustive or completely comprehensive list of all the most frequently isolated subgingival bacteria; one could argue that, although a quorum of periodontal species were not present, a diverse mixture of Gram-positive and Gram-negative, aerobe, facultative anaerobe and anaerobe were

identified to enable one to claim that these species represent more than a fair approximation of the periodontal microbiota, when cultivated *in vitro.*

16S rRNA analysis proved to be useful throughout the course of this study, enabling identification in nearly all instances to species level, of the taxa which had been isolated in pure culture at each of the sampling points. Samples analysed on the Genetic Analyser produced sequence data; identifications were obtained by a comparative analysis of the sequence data with the databases at both the RDP and BLAST. In most instances, this proved sufficient to speciate the isolate. However, the sequence data did not prove sufficient to allow delineation of the *Streptococcus pneumoniae* subgroup or the *Neisseria gonorrhoeae* subgroup, both BLAST and RDP could not resolve the sequence data, resulting in the provision of multiple matches. Indeed, the provision of insufficient sequence data is one of the possible associated problems with PCR based rRNA analysis for some closely related taxa. The problem associated with multiple matches may be ameliorated, certainly in the case of the *S. pneumoniae* subgroup, via the identification and sequencing of targets other than the 16S rRNA gene which permit the speciation of isolates. Work carried out by Poyart et al., (1998) utilised a PCR based assay to characterise an internal fragment (*sod*A) representing c.85% of the genes encoding the manganese-dependent superoxide dismutase in various streptococcal type strains. The *sod*A fragments yield an evolutionary tree having a topology similar to that of the tree constructed with full 16S rRNA sequences except there is enough sequence heterogeneity to

resolve to beyond species level (Poyart et al., 1998). Although the problems associated with speciating isolates based upon 16S rRNA analysis prompts a more fundamental question as to what precisely a bacterial species is. The advent of molecular techniques indicates that within a bacterial species one may find differing 'ecotypes', suggesting that perhaps a named species has the properties of a genus rather than a species (Cohan., 2002).

The use of a culture-based approach ensured that only a percentage of the probable population generated within the CDFF was actually catalogued. Spirochaetes may represent up to 50% of the detectable microbiota in subgingival plaque from patients with acute necrotizing ulcerative gingivitis and chronic adult periodontitis (Tanner et al., 1994) yet they were not detected as being present within the generated biofilms in this study, perhaps the molecular analysis of biofilms directly, without a culture stage may have enabled the detection of 'uncultivable' bacterial taxa.

Several culture-independent studies have been carried out on human subgingival plaque in an attempt to definitively determine the precise bacterial population contained within subgingival plaque. Perhaps one of the most extensive which has been carried out to date detected 347 different species and went on to estimate a possible 415 different species being present within subgingival plaque (Paster et al., 2001). Archeal rDNA has been identified from subgingival dental

plaque via PCR amplification and sequence analysis (Kulik et al., 2001). Perhaps we are now on the cusp of definitively determining the microorganisms present within the oral cavity.

The results of this investigation have shown that it is possible to cultivate bacterial species representative of those present in subgingival plaques associated with periodontitis within a CDFF, the consortia cultivated contained aerobic, facultative anaerobes and anaerobic bacteria. The biofilms developed from a microbiota dominated by Gram-positive aerobic/facultative anaerobes through successional events to one containing increasing number of Gram-negative anaerobic bacteria. In the next part of this study, the investigation will proceed to determine the efficacy of TBO and the antimicrobial chlorhexidine digluconate, against CDFF-cultivated biofilms, derived from pooled subgingival plaque.

CHAPTER NINE

SUSCEPTIBILITY TO LETHAL PHOTOSENSITISATION OF CDFF CULTIVATED SUB-GINGIVAL BIOFILMS

9.1 Introduction

In the previous three chapters the microcosm biofilms cultivated within the CDFF were analysed using a polyphasic approach; this demonstrated that the biofilms generated within the CDFF may be considered representative of those present in subgingival plaques associated with periodontitis, the consortia cultivated were representative microcosms, displaying many of the characteristics and composite microflora that have been associated with oral biofilms *in vivo.* Having established that the biofilms cultivated within the CDFF constitute an accurate approximation of the biofilms associated with periodontal disease, the next logical progression was to determine the effect of lethal photosensitisation upon these biofilms. The prevalence of oral diseases worldwide is often due to dental plaque (biofilm) proliferation, and the potentially pathogenic organisms which often comprise these biofilms (Hardie., 1992) therefore, when determining the efficacy of lethal photosensitisation against biofilms, a close approximation of the situation *in vivo* was important, to reinforce the credibility of any findings. In addition to subjecting the biofilms to lethal photosensitisation it was thought desirable to compare the effectiveness of lethal photosensitisation on these biofilms with an established adjunctive antimicrobial routinely prescribed for periodontal diseases. One of the most frequently prescribed therapeutic agents is chlorhexidine digluconate, this is the perceived 'gold standard', and is employed in the treatment and prevention of a wide range of oral infections (Jorgensen et al., 2001).

9.2 Materials and Methods

The CDFF was set up and inoculated as described in chapter 6, section 6.2

9.2.1 Sampling of the CDFF

This was carried out as described in section 2.2.7. The microcosm biofilms cultivated within the CDFF were grown for 7 days, to allow the biofilms to approach maturation before being extracted for lethal photosensitisation or determinations as to the susceptibility of the microcosm biofilms to chlorhexidine digluconate.

9.2.2 Preparation of TBO

This was carried out as described in chapter 2, section 2.6.1.1

9.2.3 Susceptibility of CDFF-grown multi-species biofilms to TBO/HeNe light

This was carried out as described in chapter 2, section 2.6.4.

9.2.4 Susceptibility of CDFF grown multi-species biofilms to chlorhexidine

Two PTFE pans containing 5 hydroxylapatite discs each were removed aseptically from the CDFF and placed in a sterile universal; 5.0 ml of 0.2 % chlorhexidine digluconate (CHG) (Sigma Ltd.) (0.2 % CHG was utilised due to its frequent use in mouthwashes) or, in the case of the control pan, 5.0 ml of phosphate buffered saline (PBS) (Oxoid Ltd.) was added to the universal and

incubated for the requisite period of time. Three discs per treatment (n=6) were then prepared for viable counting. Ten-fold serial dilutions were prepared in BM broth. Duplicate 25 µl aliquots were then spread over the surface of FAA plates. After incubation in an anaerobic cabinet for up to 7 days, the colonies on the appropriate plate were subsequently counted.

9.2.5 CLSM of CDFF cultivated biofilms and subsequent analysis

The method used was the same as that described in chapter 2, section 2.5.

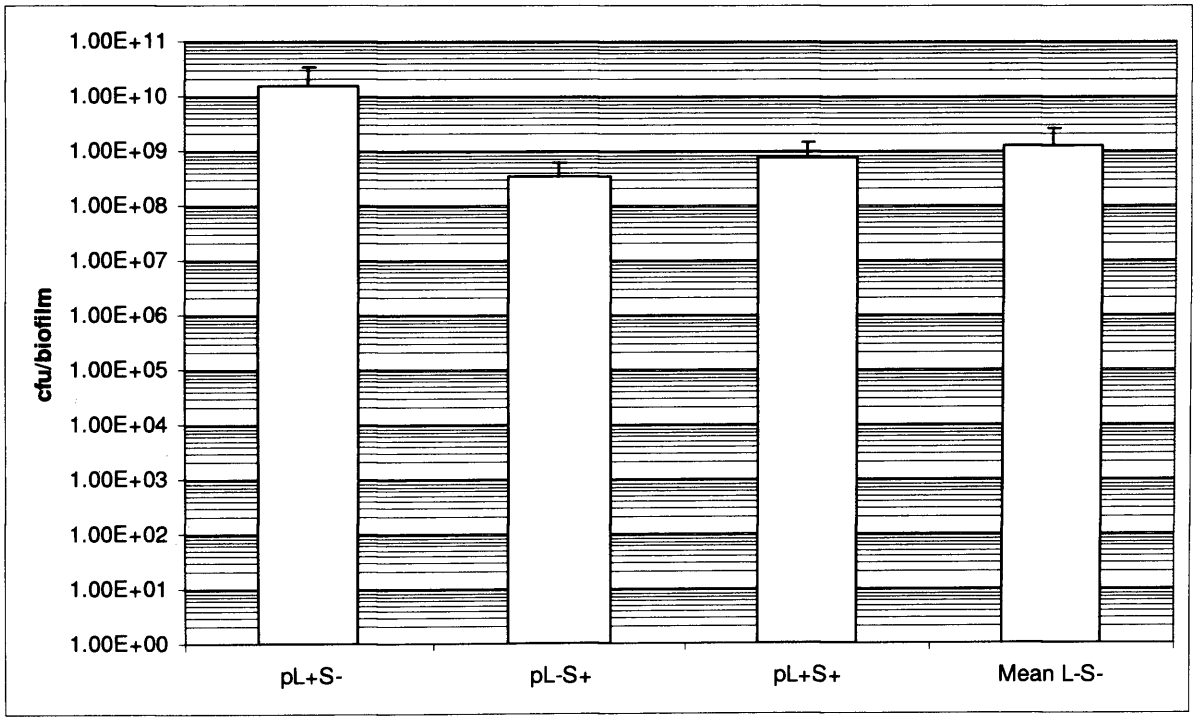
9.2.6 Statistical analysis of the data

The statistical significance of the data was ascertained using the two-tail t-test assuming unequal variance.

9.3 Results

After cultivation of the biofilms within the CDFF for at least 7 days (to allow the biofilms to fully form and mature), pans containing biofilms were removed from the CDFF and subjected to lethal photosensitisation using varying concentrations of TBO and differing HeNe laser light doses.

Figure 9.1: Viable counts of CDFF cultivated multi-species biofilms exposed to 63 J of HeNe laser light (energy density, 163.7 Jcm^{-2}) in the presence of 10 µl of 32.7 µM pTBO with appropriate controls.



pL+S+: Exposure to both laser light and pharmaceutical grade photosensitiser

pL+S-: Exposure to laser light but not to pharmaceutical grade photosensitiser

pL-S+: Exposure to pharmaceutical grade photosensitiser but not to laser light

mean L-S- : Exposure to neither laser light nor pharmaceutical grade photosensitiser (Two control discs C1 and C2)

As is seen in figure 9.1 subjection of multi-species biofilms to lethal photosensitisation resulted in a mere 41.9 % reduction in the number of recovered viable organisms (L+S+) compared to the control biofilms (mean L-S-). However, photosensitiser in the absence of laser light (L-S+) resulted in a 72.6 % reduction in the number of recovered viable organisms, this reduction was not found to be statistically significant ($P = 0.08$).

Figure 9.2: Viable counts of CDFF cultivated multi-species biofilms exposed to 63 J of (energy density, 163.7 Jcm^{-2}) HeNe laser light in the presence of 10 µl of 81.7 µM pTBO with appropriate controls.

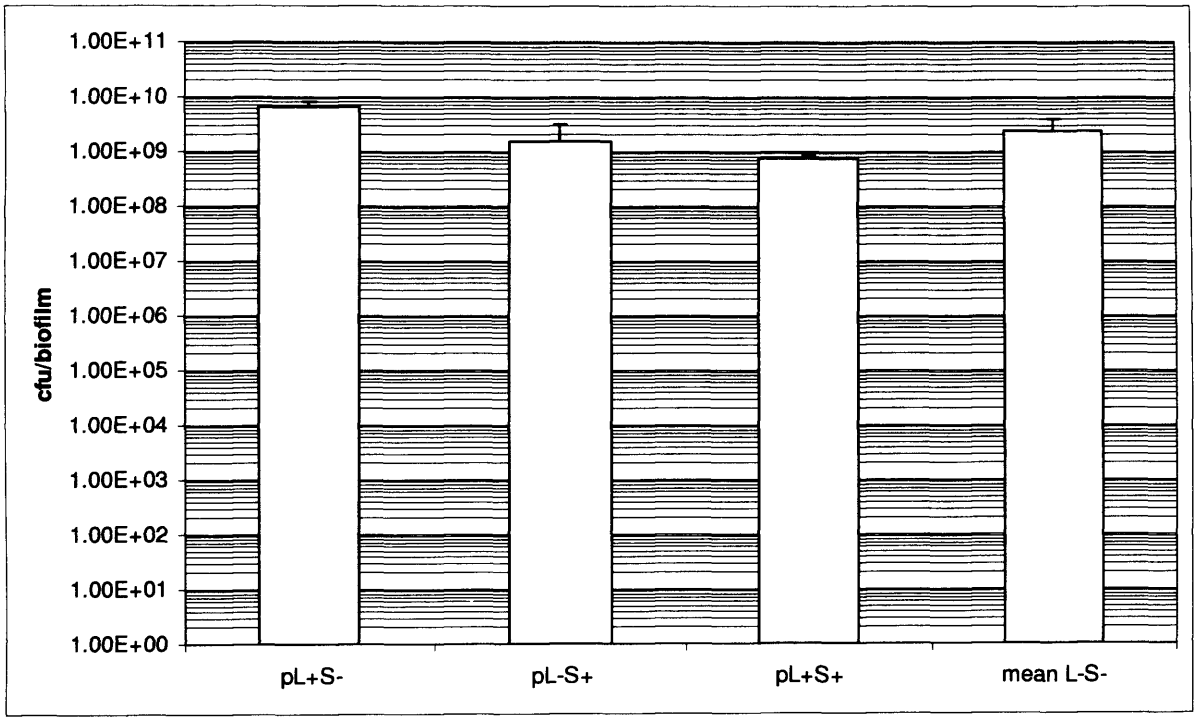


Figure 9.2 shows that exposure of the CDFF grown biofilms to 81.7 µM of pTBO with 63 J of HeNe laser light resulted in a 69% reduction in the number of recovered viable organisms (L+S+) compared to the control discs (Mean L-S-), this was found to be statistically significant (P = 0.02). However there was also a 36.3 % reduction in the number of recovered viable organisms elicited by the photosensitiser alone (L-S+), this was not found to be statistically significant (P = 0.43)

Figure 9.3: Viable counts of CDFF cultivated multi-species biofilms exposed to 94.5 J of HeNe laser light (energy density, 245.6 Jcm^{-2}) in the presence of 10 µl of 32.7 µM pTBO with appropriate controls.

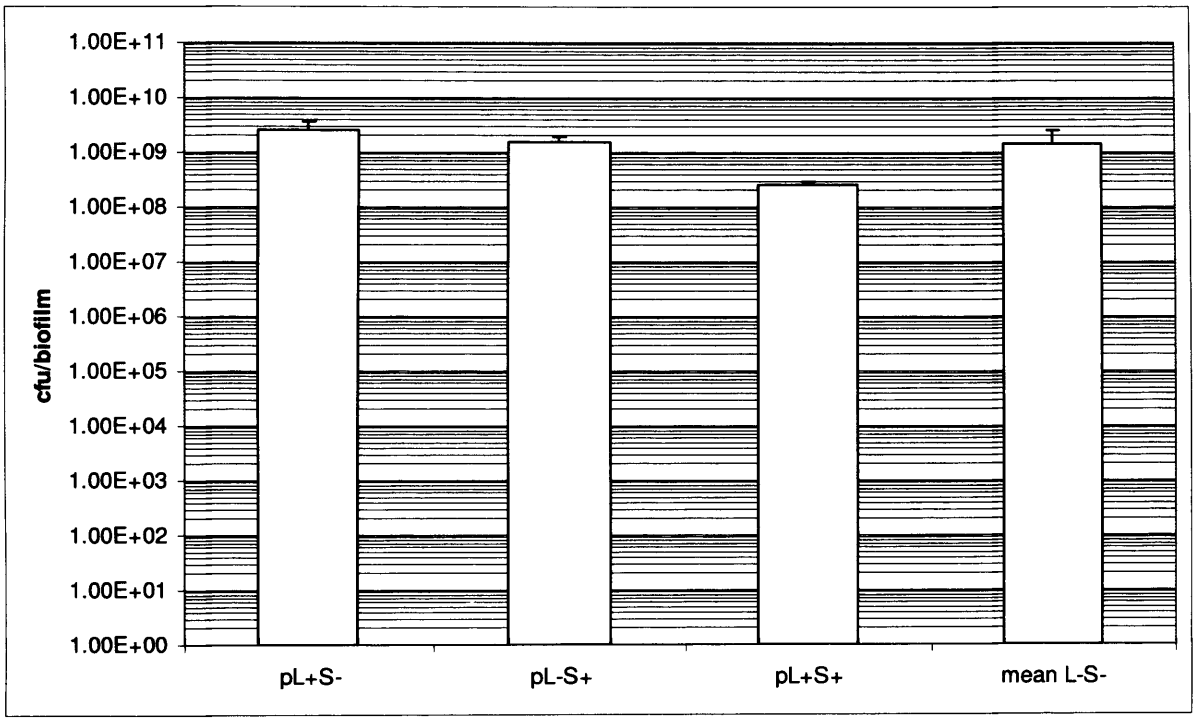
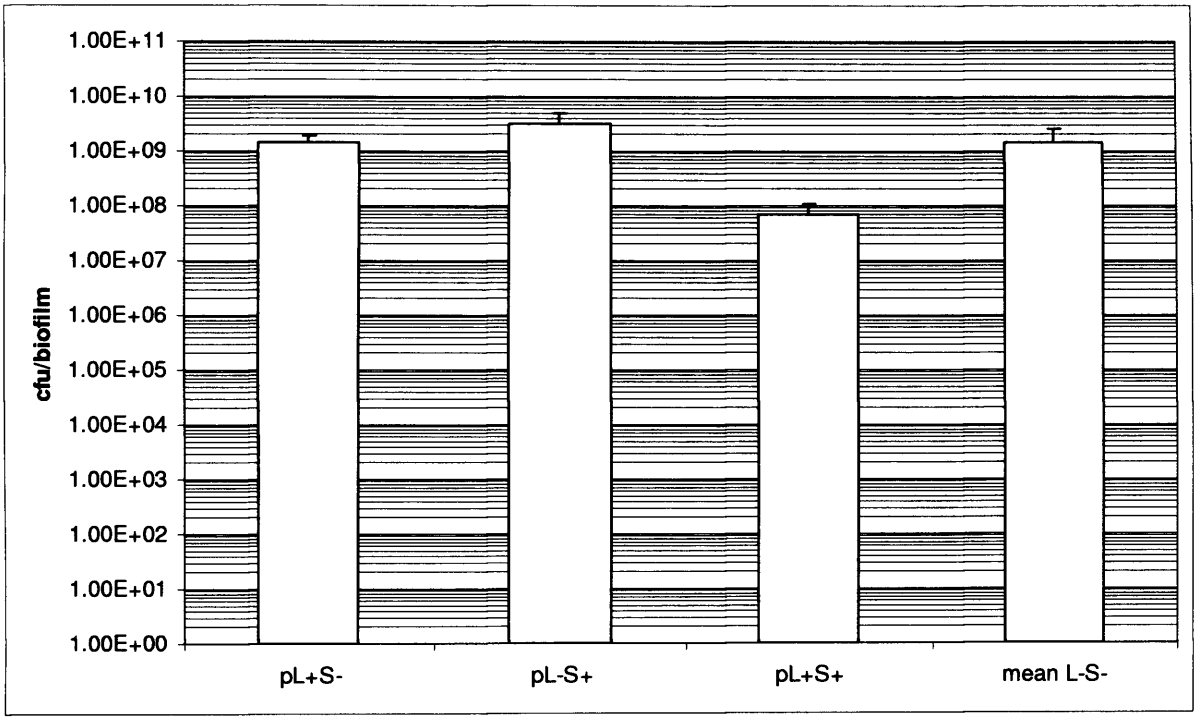


Figure 9.4 shows that exposure of CDFF-cultivated multi-species biofilms to10 µl of 32.7 µM pTBO when illuminated with 94.5 J of laser light produced a statistically significant 82.7% reduction in recovered viable bacteria (L+S+) (P = 0.02) when compared to the control discs (mean L-S-).

Figure 9.4: Viable counts of CDFF cultivated multi-species biofilms exposed to 94.5 J of HeNe laser light (energy density, 245.6 Jcm^{-2}) in the presence of 10 µl of 65.4 µM pTBO with appropriate controls.



Exposure of multi-species biofilms to lethal photosensitisation under experimental conditions as outlined in figure 9.5 resulted in a 95% reduction in the number of recovered viable organisms (L+S+), compared to the control discs (Mean L-S-). This was found to be statistically significant (P = 0.01).

Figure 9.5: Viable counts of CDFF cultivated multi-species biofilms exposed to 94.5 J of HeNe laser light (energy density, 245.6 Jcm^{-2}) in the presence of 10 µl of 81.7 µM pTBO with appropriate controls.

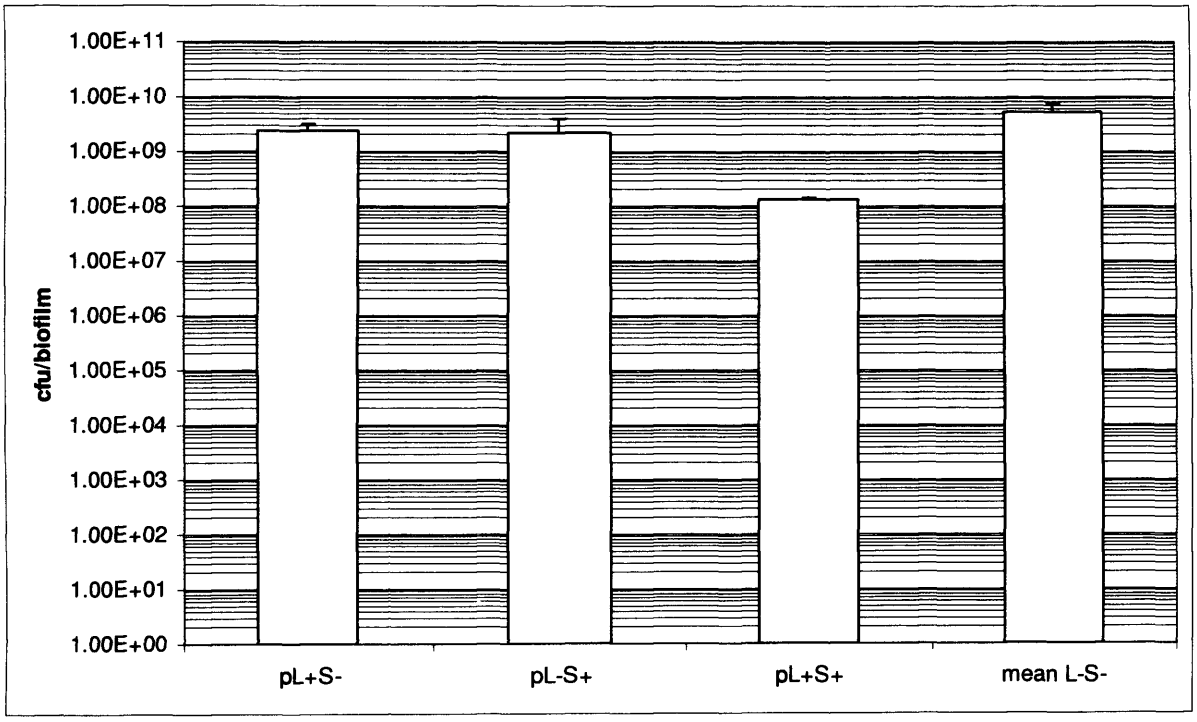


Figure 9.6 shows that exposure of CDFF-cultivated multi-species biofilms to 94.5 J of HeNe laser light in the presence of 10 µl of 81.7 µM pTBO produced a statistically significant 97.4 % reduction (P = 0.0009) in recovered viable bacteria (L+S+) when compared to the control discs (mean L-S-). However a 54.4 % reduction in recovered viable bacteria was obtained by laser light in the absence of TBO (L+S-) and a 57.1 % reduction in recovered viable bacteria by pTBO in the absence of laser light (L-S+), both of these reductions were found to be statistically significant (L+S- P= 0.01, L-S+ P = 0.04).

Figure 9.6: Viable counts of CDFF cultivated multi-species biofilms exposed to 94.5 J of HeNe laser light (energy density, 245.6 Jcm^{-2}) in the presence of 10 µl of 163.4 µM pTBO with appropriate controls.

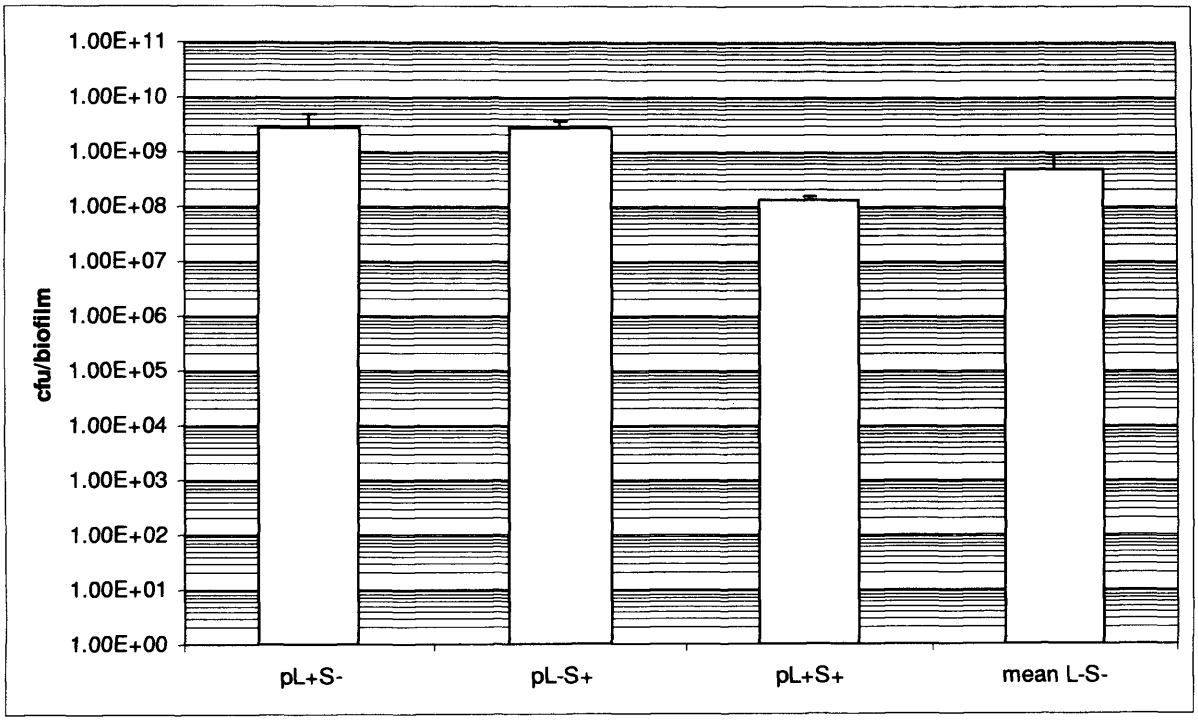
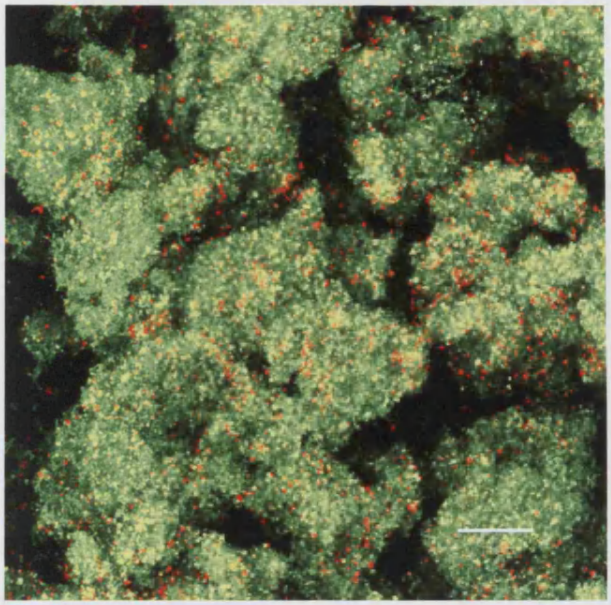


Figure 9.7 shows that exposure of multi-species biofilms to lethal photosensitisation produced a 74.4 % reduction in recovered viable bacteria (L+S+) when compared to the control discs (mean L-S-). This was found to be statistically significant (P = 0.04). It appeared that increasing the administered light dose or varying photosensitiser concentration applied to the biofilms did not correlate with a decrease in the number of recovered viable organisms post-exposure (L+S+), at least for the light doses and photosensitiser concentrations investigated in this study.

Figure 9.7a: a confocal micrograph of a control (L-S-) CDFF-cultivated multi-species biofilm, the bar represents 20 µm.



The control biofilm shown in figure 9.7a may be considered an archetypal biofilm. Clearly visible were the microcolony formations and the water channels interspersing these microcolonies, qualitatively the viable cells (stained green) appeared to be far more numerous than the nonviable cells (stained red). Figure 9.7b shows another CDFF-cultivated multi-species biofilm that was subjected to lethal photosensitisation. Visually there was a discernible difference between this biofilm and the pristine biofilm in figure 9.7a. The structure of the biofilm seems to have been disrupted by the lethal photosensitisation. In addition, qualitatively, there appeared to be larger numbers of nonviable cells when compared to viable cells.

Figure 9.7b: a confocal micrograph of a CDFF cultivated multi-species biofilm having been exposed to 63 J of HeNe laser light (energy density, 163.7 Jcm^{-2}) in the presence of 10 µl of 81.7µM pTBO. The bar represents 20 µm

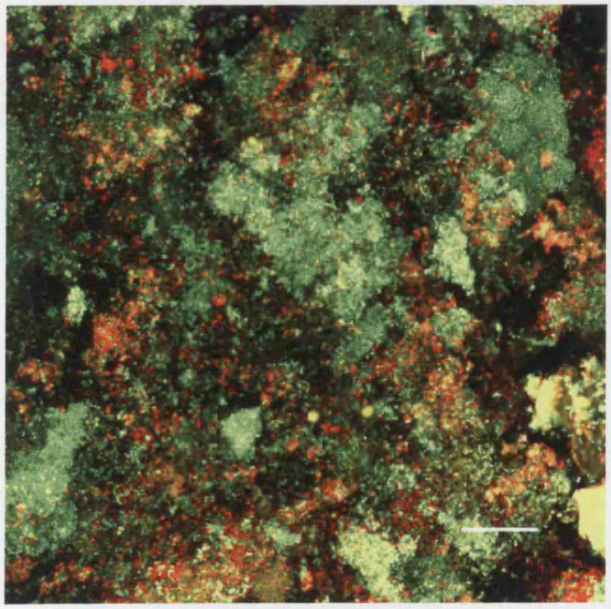
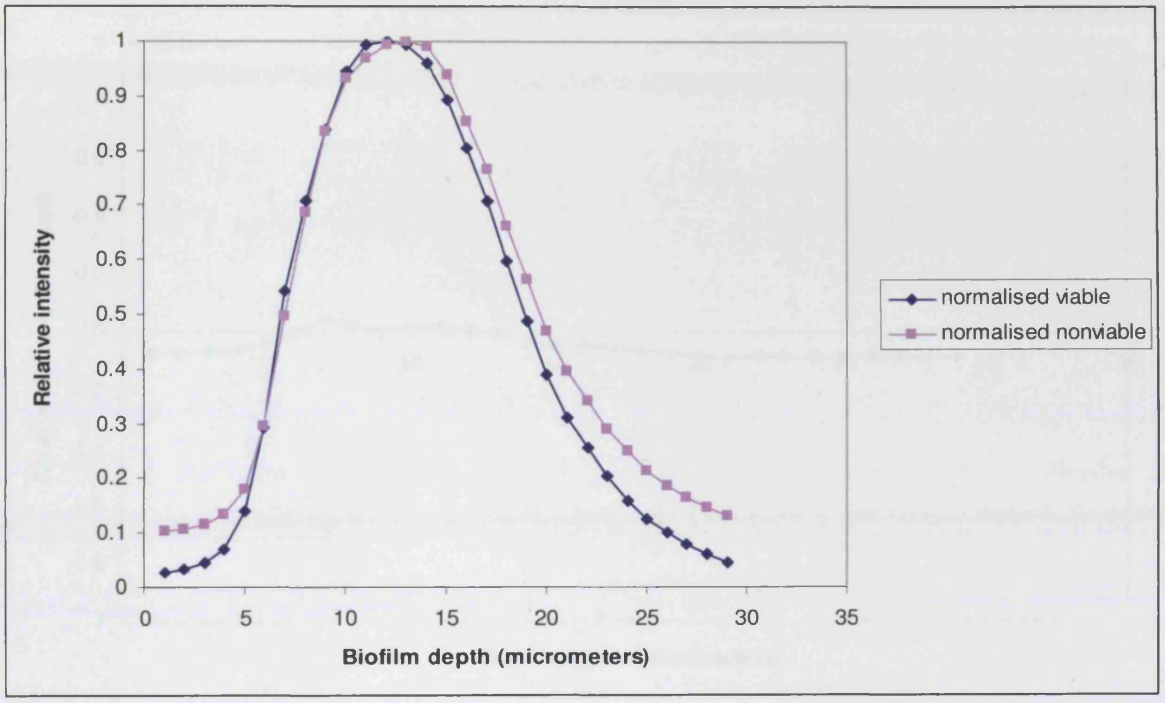


Figure 9.8a: The depth into a control (L-S-) CDFF cultivated multi-species biofilm as determined by CLSM versus the normalised image intensity for both the viable and nonviable channels.



As is evident from figure 9.8a, that the distribution of both viable and nonviable cells through the z plane of the CDFF-cultivated biofilms was extremely similar. This demonstrated that within pristine biofilms there appeared to be comparable numbers of live and dead organisms, the exceptions seemingly being on the periphery of the biofilm, proximal to the biofilm substratum interface and at the biofilm surface interface where there were seemingly higher numbers of nonviable than viable cells.

Figure 9.8b: The changes in the viable to nonviable relative image intensity (using normalised data) as a function of biofilm depth in a control (L-S-) CDFF cultivated multi-species biofilm as determined by CLSM.

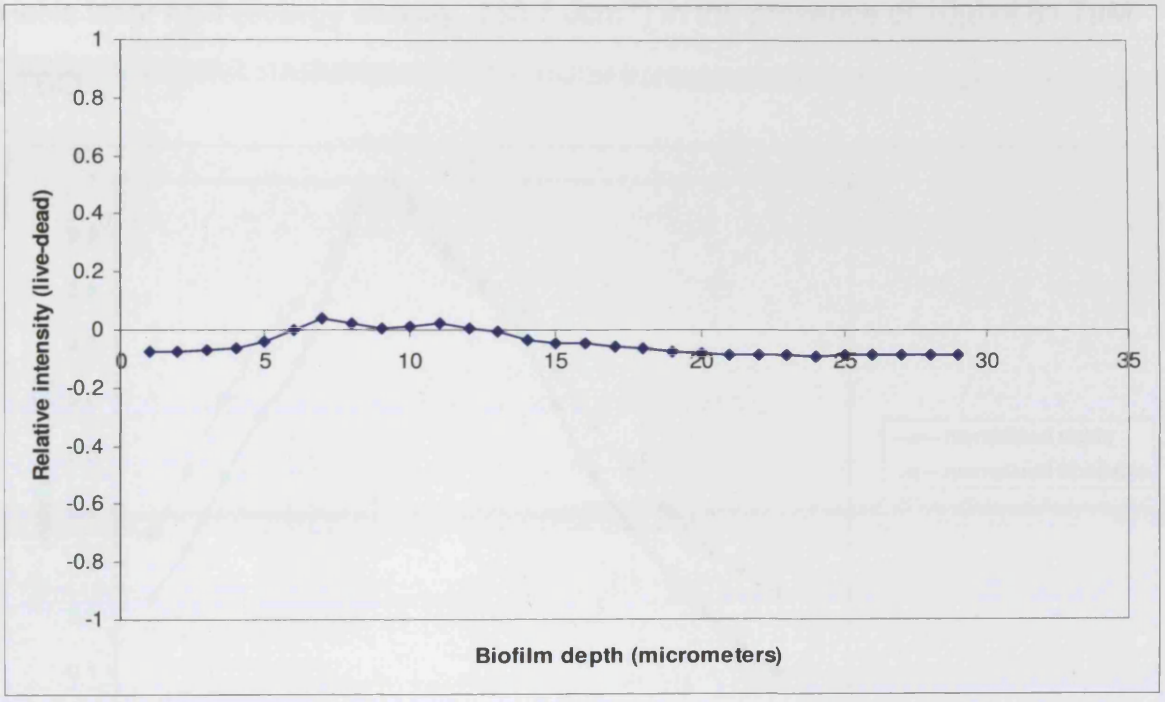


Figure 9.8b shows the relative numbers of viable to nonviable cells throughout the z plane of the biofilm. Quite clearly visible was the trend that was identifiable in figure 9.8a, nonviable cells being more numerous on the periphery of the biofilm, although there were not appreciably larger numbers of nonviable than viable cells.

Figure 9.9a: The depth into a photosensitised (L+S+) CDFF cultivated multi-species biofilm as determined by CLSM versus the normalised image intensity for both the viable and nonviable channels. Biofilms were exposed to 63 J of HeNe laser light (energy density, 163.7 Jcm^{-2}) in the presence of 10µl of 81.7µM pTBO.

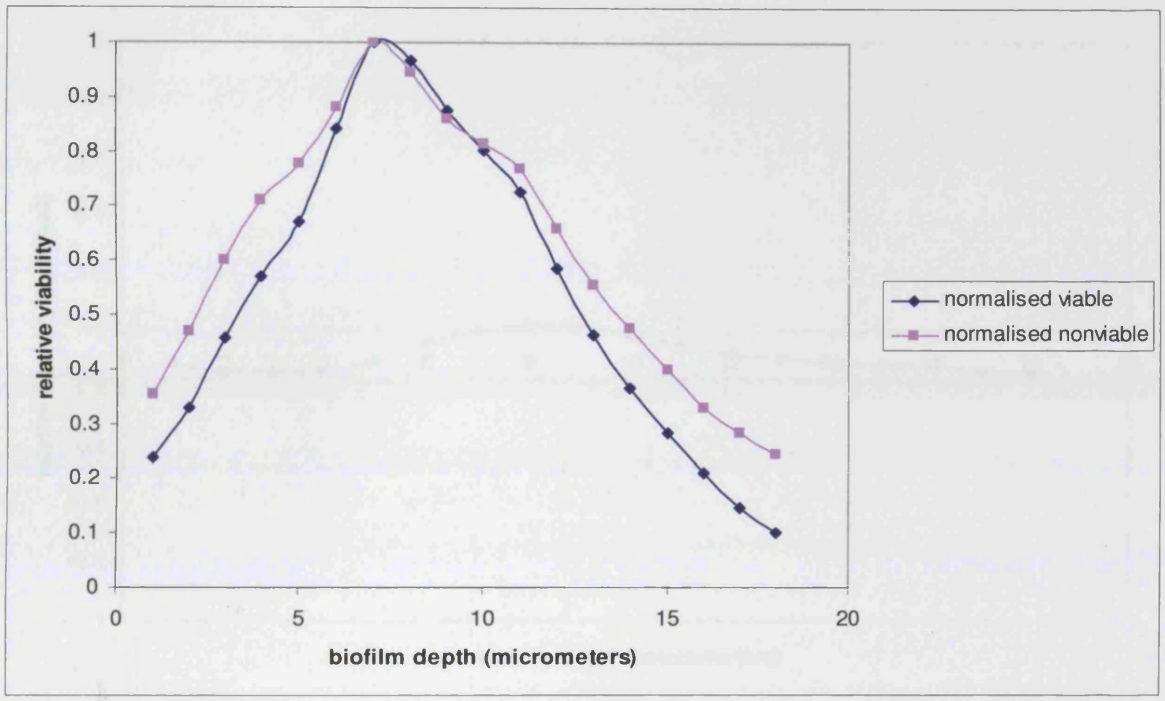
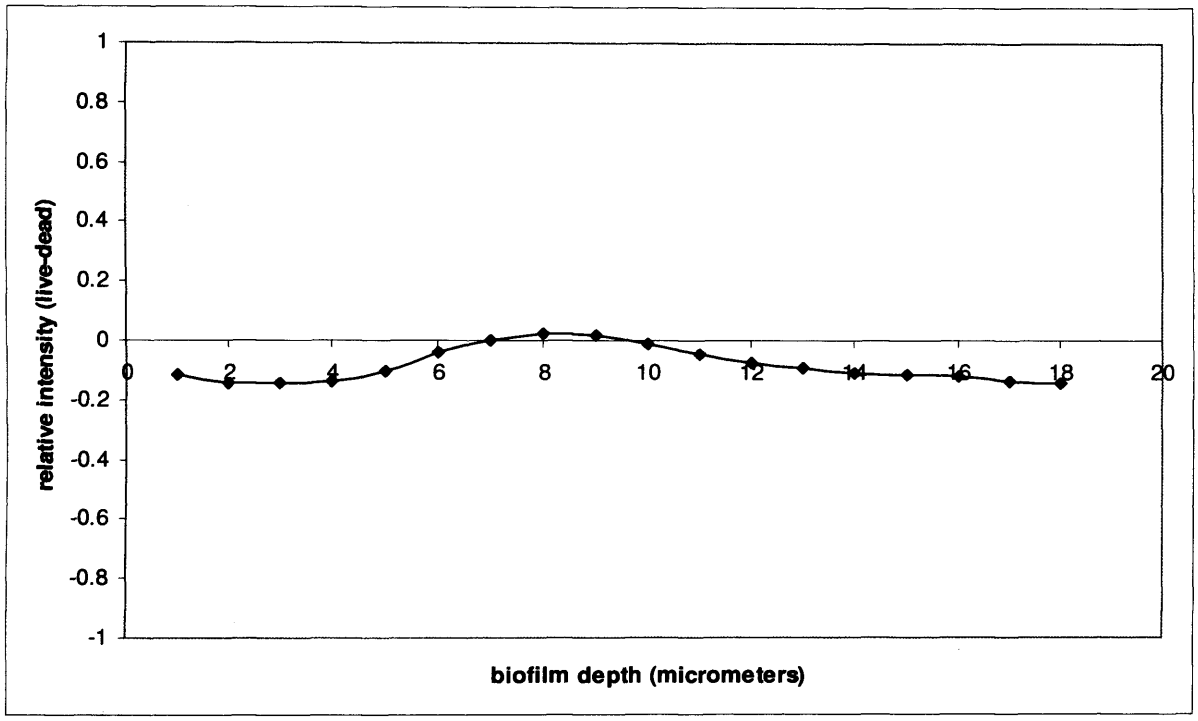


Figure 9.9a shows the normalised values for both the viable and nonviable channels throughout the z plane of the biofilm. Interestingly, throughout the z plane of the biofilm there was a higher normalised image intensity for the nonviable channel than the viable channel. This mimicked what was evident in the pristine biofilm (L-S-), however, this trend appears to be rather more pronounced in the photosensitised biofilm.

Figure 9.9b: The changes in the viable to nonviable relative image intensity (using normalised data) as a function of biofilm depth in a photosensitised (L+S+) CDFF-cultivated multi-species biofilm as determined by CLSM. Biofilms were exposed to 63 J of HeNe laser light (energy density, 163.7 Jcm^{-2}) in the presence of 10µl of 81.7µM pTBO.

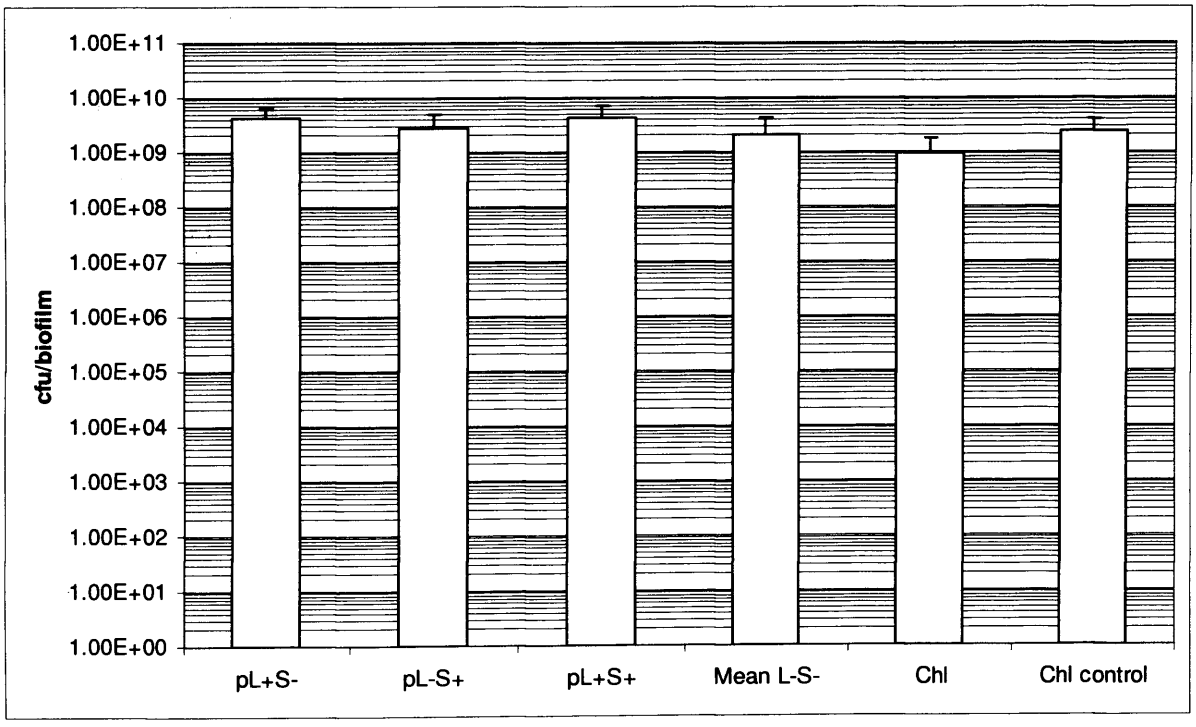


In figure 9.9b is seen the relative image intensity of viable to nonviable cells throughout the z plane of the CDFF-cultivated multi-species biofilm. What was evident was the preponderance of nonviable cells proximal to the biofilm surface interface and the biofilm surface interface, the largest concentration of viable cells was found equidistant from the peripheries of the biofilm, concentrated roughly midway through the z plane of the biofilm. It was also apparent that lethal photosensitisation resulted in contraction of the biofilm as the

photosensitised biofilm was only 18 μm in depth compared to c.30 μm for the pristine biofilm.

In addition to subjecting the CDFF cultivated biofilms to lethal photosensitisation, the effect of chlorhexidine digluconate upon the biofilms was investigated. This was then compared to the effect of lethal photosensitisation upon the biofilms under similar experimental conditions (i.e. the contact time between biofilm and chlorhexidine digluconate was the same as the exposure time of biofilm to lethal photosensitisation).

Figure 9.10: Viable counts of CDFF cultivated multi-species biofilms exposed to 2.1 J of HeNe laser light (energy density, 21.8 Jcm^{-2}) in the presence of 10 μl of 81.7 μM pTBO and CDFF cultivated multi-species biofilms exposed to 0.2 % chlorhexidine digluconate for 1 minute with appropriate controls.



pL+S+: Exposure to both laser light and pharmaceutical grade photosensitiser

pL+S-: Exposure to laser light but not to pharmaceutical grade photosensitiser

pL-S+: Exposure to pharmaceutical grade photosensitiser but not to laser light

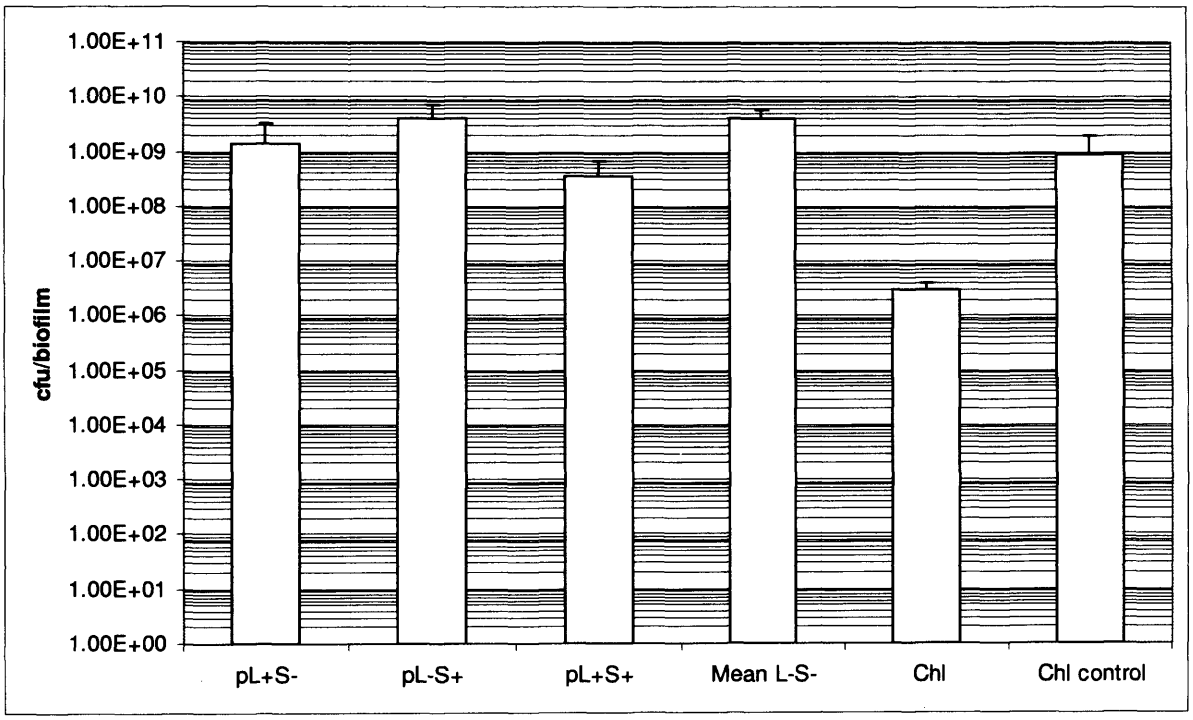
mean L-S- : Exposure to neither laser light nor pharmaceutical grade photosensitiser (Two control discs C1 and C2)

Chl: Exposure to 0.2 % chlorhexidine digluconate for 1 min.

Chl control: Exposure to PBS for 1 min.

As may be seen in figure 9.10, exposure of CDFF-cultivated biofilms to 2.1 J (1 min exposure) of HeNe laser light in the presence of 10 µl of 81.7 µM pTBO did not result in a statistically significant reduction in the number of recovered viable organisms (L+S+) when compared to the control discs (Mean L-S-). When the multi-species biofilms were exposed to 0.2 % chlorhexidine digluconate for 1 min this treatment did not result in a statistically significant reduction in the number of recovered viable organisms post-exposure when compared to the control. Hence, it appears that both chlorhexidine digluconate and lethal photosensitisation are equally ineffectual when the contact time is limited to 1 min.

Figure 9.11: Viable counts of CDFF cultivated multi-species biofilms exposed to 31.5 J of HeNe laser light (energy density, 81.9 Jcm^{-2}) in the presence of 10 µl of 81.7 µM pTBO and CDFF cultivated multi-species biofilms exposed to 0.2 % chlorhexidine digluconate for 15 min with appropriate controls.



As may be seen in figure 9.11 exposure of CDFF cultivated biofilms to 31.5 J (15 min exposure) of HeNe laser light in the presence of 10 µl of 81.7 µM pTBO resulted in a statistically significant 90.8 % ($P = 0.0003$) reduction in the number of recovered viable organisms (L+S+) when compared to the control discs (Mean L-S-). When the multi-species biofilms were exposed to 0.2 % chlorhexidine digluconate for 15 min this treatment resulted in a 99.7 % reduction in the number of recovered viable organisms post-exposure when compared to the control, however this was not statistically significant due to appreciable variation in the numbers of cells enumerated from the control biofilms. Both lethal

photosensitisation and application of 0.2 % chlorhexidine digluconate resulted in large reductions in the number of viable organisms recovered post-exposure when applied for 15 minutes.

Discussion

After the comprehensive analysis of the subgingivally-derived biofilms generated within the CDFF in the preceding three chapters, the natural progression was to utilise the CDFF to generate biofilms which may then be used to determine the efficacy of TBO in combination with HeNe laser light and, for comparative purposes, the antimicrobial chlorhexidine digluconate on the biofilms. The data presented in this part of the investigation consisted solely of total counts derived from FAA plates incubated anaerobically. However, when this investigation was originally performed, supplementary data were generated using a range of selective media; CFAT to screen for *Actinomyces* spp., *Veillonella* spp. on Veillonella agar, streptococci on MS agar and Gram-negative anaerobic species with FAX. When all of these data were processed the resultant histogram became rather confused, there was a profusion of information, making comprehension of the data almost impossible; to apply a metaphor it became difficult to 'see the wood for the trees'. Hence the decision was taken to omit the data generated using the selective media. If an appreciable antimicrobial effect was being exerted then this should be reflected in a discernible reduction in the number of recovered viable organisms (total anaerobic count) when compared to the control biofilms.

The results presented in this chapter demonstrate that exposure of the CDFF-grown biofilms to HeNe laser light in the presence of TBO resulted in lethal photosensitisation of the CDFF-cultivated multi-species biofilms. A *prima facie* analysis of the data does not reveal any glaringly obvious trends. Two light dose regimes were employed, 63 J and 95.4 J, and the concentration of TBO applied was also varied, ranging from 32.7 μM to 163.4 μM. The first experimental results (figure 9.1) showed that application of TBO at a concentration of 32.7 μM in conjunction with a light dose of 63 J resulted in a 41.9 % reduction in the number of recovered viable organisms (L+S+) which was not significant. However when photosensitiser concentration was increased to 81.7 μM (figure 9.2), keeping all other experimental parameters unchanged, a concomitant decrease in the number of recovered viable organisms was recorded, 69 % (L+S+), this did prove to be significant. The data presented in figures 9.3 – 9.6 involved increasing the light dose administered from the 63 J used previously to 94.5 J, the concentration of photosensitiser applied was increased progressively through the series of experiments. The initial photosensitiser concentration used at the increased light dose was 32.7 μM (figure 9.3) which resulted in a 82.7 % reduction in the number of recovered viable organisms (L+S+), which was significant. Upon increasing the sensitizer concentration to 65.4 μM (figure 9.4) a concomitant decrease in recovered viable organisms was observed, a 95 % kill (L+S+), again this was significant. When the concentration of TBO was again increased to 81.7 μM (figure 9.5) an associated increase in kill to 97.4 % was recorded. Finally, the concentration of TBO was doubled from that used

previously to 163.4 μM (figure 9.6). Whilst a significant kill was recorded, this proved to be lower than that observed previously, the dosimetric effect seen previously was not evident, confirming that simply increasing the concentration of photosensitiser did not correlate with a concomitant increase in kill in all instances. From the data generated, it was possible to draw some tentative conclusions. Firstly, it seemed that increasing the light dose administered from 63 J to 94.5 J resulted in an observable increase in kill. When comparable data sets are compared, for example figures 9.1 and 9.3 it appeared that increasing the light dose administered whilst maintaining all other experimental parameters did indeed result in an increase in kill. This same trend was apparent when comparing figures 9.2 and 9.5, an appreciable increase in efficacy was achieved merely by increasing the light dose administered. Secondly, it appeared that increasing the concentration of photosensitiser applied also resulted in an increase in kill, the caveat being that photosensitiser concentration seemed to only increase the kill up to a point, any increase in photosensitiser concentration past this point did not result in an increased kill. This observation seemed somewhat counter-intuitive; one would have envisioned that by merely increasing the concentration of photosensitiser applied to the biofilm would have resulted in an observable increase in kill. As the concentration of TBO was increased so the intensity of the resultant blue colouration also increased; if the concentration of TBO used was not 'sufficient' for the light dose administered photo-bleaching would occur, conversely if the TBO solution was quite concentrated, penetration of the solution by the laser light proved to be rather difficult. This produced a

shielding effect, preventing the excitation of TBO proximal to the biofilm bacteria, reducing the kill obtained. Hence, there appears to be a concentration of TBO optimal for photosensitisation, whereby photo-bleaching does not completely degrade the TBO present but photosensitiser concentration is not sufficient to retard laser light penetration.

As noted in chapter 8, the bacterial composition of the biofilms cultured within the CDFF did vary somewhat from run to run. This variability also extended to differences in composition of differing biofilms within the same run. These compositional differences encompassed small variations in the absolute number of bacteria present from biofilm to biofilm and differences in the species composition from biofilm to biofilm. Whilst such variations, however small, are less than ideal, they represent an experimental reality. Any reduction in the number of recovered viable bacteria, be it in the controls or in the biofilm being subjected to lethal photosensitisation, must be tempered by the fact that this may have been due, at least in part, to slightly differing numbers of viable organisms composing the biofilms at the commencement of the experiment. Fluctuating numbers of organisms present from biofilm to biofilm was also a problem encountered with biofilm cultivation upon membrane filters (chapter 5); hence it appears that variations in biofilm composition are unavoidable, regardless of the model used to generate them.

The photodynamic effect of pharmaceutical grade TBO upon CDFF-cultivated biofilms was determined within this part of the investigation. The effect of Sigma grade TBO upon CDFF cultivated biofilms was not determined due to the results generated during chapters 3 – 5, the preliminary investigations. The conclusions drawn suggested that biofilm-grown bacteria possess an inherent recalcitrance to the photodynamic action of TBO in conjunction with HeNe laser light, especially when compared to planktonic oral organisms. However, the collective lesson drawn from these chapters was that susceptibility to TBO-mediated photoinactivation was not dependent upon whether the TBO happened to be Sigma or pharmaceutical grade, it was consistently demonstrated that the kill achieved did not differ due to the Grade of TBO used. Hence, throughout this part of the investigation, pharmaceutical grade TBO was used, due to no difference in efficacy being evident between the two grades of photosensitiser in the initial investigations.

Previous work has been carried out by Wood et al., 1999 utilising a cationic phthalocyanine (pyridinium Zn phthalocyanine) in conjunction with white light, to photosensitise natural plaque biofilms formed *in vivo*. These biofilms were generated by the implantation of an *in situ* device into the oral cavity of volunteers for a period of seven days before being extracted. This contrasts with the *in vitro* approach adopted for this study. However, despite the differences in methodology as regards biofilm generation and a differing photosensitising system, there seems to be a broad consensus between their results and

observations recorded during this study. For instance, following photosensitisation of the biofilms and analysis by confocal microscopy no selectivity of photosensitisation was observed, with extensive photosensitisation evident (Wood et al., 1999). This tallies with what was observed following lethal photosensitisation (figure 9.7b), where killing appears to be wide-spread, illustrating the effectiveness of lethal photosensitisation, although despite the high prevalence of non-viable cells evident, the distinct microcolony formations do not appear to have completely disintegrated. Additionally, Wood et al., (1999) also found that following photosensitisation the biofilm contracts such that photosensitised biofilms are approximately half the thickness of the controls. This phenomenon was also evident during this study, if figures 9.8b and 9.9b are compared it is apparent that lethal photosensitisation results in biofilm contraction. This physical contraction of the biofilm following lethal photosensitisation is clearly beneficial, the outcome being two-fold; the killing of biofilm bacteria and a reduction in plaque thickness which amounts to a form of atraumatic scaling (Wood et al., 1999). Perhaps this contraction in biofilm thickness may be due, in part, to the deterioration of the microcolony structures brought about by lethal photosensitisation. It is the distinctive microcolony motif which provides the structural scaffold of the biofilm, forming the system of channels which intersperse biofilms providing nutrients and removing metabolic waste-products. When one views a biofilm that has been subjected to lethal photosensitisation and sees the damage inflicted to the microcolony structures, it is perhaps unsurprising that this results in a concomitant reduction in biofilm

thickness. Moreover, the microcolony structures characteristic of biofilms may help to explain the observed recalcitrance of biofilm-grown bacteria to lethal photosensitisation, certainly when compared to planktonic oral organisms. Biofilms are composed of microbes surrounded by a complex coating, an accretion consisting primarily of polysaccharides (Sutherland, 2001, Wilson, 2001). It is reasonable to propose that the photosensitiser, especially a water soluble, cationic photosensitiser such as toluidine blue would interact with the abundant EPS; hence, upon activation of the photosensitiser by HeNe laser light interaction of the resultant radicals with the EPS is much more probable that diffusion and interaction with vulnerable cellular targets, such as the cytoplasmic membrane of a Gram-negative bacterium. Hence, the physical propinquity of the photosensitiser to the abundant EPS and associated organic molecules would act to quench the activated photosensitiser before it could come into contact with a bacterium (Wilson et al., 1993b, Wood et al., 1999).

Photodynamic therapy as a treatment modality for periodontal diseases is still very much in its infancy. Hence, it was desirable to gauge the efficacy of the photosensitisation system used in this study with an adjunctive antimicrobial compound such as chlorhexidine digluconate. As stated previously, chlorhexidine was selected for comparative purposes due to its widespread use in the treatment of a wide range of oral infections. The results obtained seem to confirm that lethal photosensitisation may be compared favourably with chlorhexidine digluconate. When the biofilms were subjected to a light dose of

2.1 J in conjunction with 81.7 μM of TBO no bacterial killing was observed, however this mimicked what was found when the biofilms were immersed in chlorhexidine digluconate for 1 minute. Both lethal photosensitization and chlorhexidine digluconate proved to be equally ineffectual when the contact time was limited to 1 minute. Increasing the contact time between the biofilms and chlorhexidine digluconate from 1 minute to 15 minutes resulted in an appreciable kill; increasing the light dose administered from 2.1 J to 31.5 J also resulted in an appreciable kill, comparable to that achieved using chlorhexidine digluconate. Hence, it is possible to conclude from these preliminary data that lethal photosensitisation using TBO in conjunction with HeNe laser light seemed to have performed equally as well as the antimicrobial chlorhexidine digluconate. The susceptibility of CDFF-cultivated multi-species biofilms to 0.2 % chlorhexidine digluconate has previously been experimentally determined (Pratten et al., 1998b). Contact times of 1, 5 and 60 min were examined; it was found that substantial reductions in bacterial viability resulted only from a 60 min exposure (Pratten et al., 1998b). This result reinforces the findings in this study exemplifying the recalcitrant nature of biofilms.

Photodynamic therapy has previously been shown to be effective in the presence of blood. Elimination of *F. nucleatum* and black-pigmented anaerobes was reported from sub-gingival plaque samples which had been exposed to HeNe laser light and TBO in the presence of 10% horse blood (Wilson et al.,1993c). This is significant as bleeding and elevated levels of GCF are often associated

with periodontal disease, and therefore if PDT is to have any utility 'in the field' it must be shown to be effective in such a milieu. For PDT to have a widespread therapeutic application it is necessary to eliminate the bacterial constituents of a periodontal lesion whilst leaving undamaged the adjacent tissue; it has been shown that application of TBO in concentrations sufficient to mediate killing of *S. sanguinis* (8.17 μM and 16.3 μM) did not produce a statistically significant reduction in the viability of human oral gingival fibroblasts and keratinocytes upon irradiation with a HeNe laser. However, it was demonstrated that TBO did display cytotoxicity to these cell lines at low concentrations (16.3 μM) (Soukos et al.,1996). Photodynamic therapy does appear to be potentially a suitable treatment of periodontal diseases. It avoids the use of systemic antibiotics and the associated problem of perturbation of the commensal microflora; whilst local application of antibiotics often proves to be discomforting to the patient being treated. The increasing prevalence of antibiotic resistance is also a stimulating factor in the development of novel treatment methodologies. It has recently been demonstrated that sub-lethal concentrations of the anti-fungal triclosan may select for resistant mutants in *Escherichia coli* (Gilbert et al., 2002). Triclosan targets the enzyme enoyl reductase which is involved in lipid biosynthesis. Hence triclosan may select for mutants in a target that is also shared with the anti-*E. coli* diazaborine compounds and the antituberculosis drug isoniazid (Gilbert et al., 2002). Indeed, sublethal concentrations of antimicrobials have also been shown to induce expression of multidrug efflux pumps and efflux mutants (Gilbert et al., 2002). Lethal photosensitisation mediates bacterial killing

via singlet oxygen and free radicals. Resistance development would be unlikely due to the multiplicity of target sites, the outer membrane and plasma membrane of Gram-negative bacteria, DNA as well as photolabile surface-associated proteins (Bhatti et al., 1998, Bhatti et al., 2001) against which the generated singlet oxygen and free radicals are active (Wilson 1993d)

The results of this investigation have shown that CDFF-cultivated biofilms are indeed susceptible to lethal photosensitisation when using TBO in conjunction with a HeNe laser. However, this susceptibility to lethal photosensitisation is very much dependent upon experimental conditions. This investigation has also shown that under comparable experimental conditions, lethal photosensitisation exerts a comparable antimicrobial effect to 0.2 % chlorhexidine digluconate.

CHAPTER TEN

FINAL DISCUSSION AND CONCLUSIONS

10 Final discussion and conclusions

The application of photodynamic therapy as a treatment modality for infectious diseases is still very much in its infancy. A plethora of *in vitro* work that has been performed over the past fifteen years has served to increase our knowledge base as to the effectiveness of various photosensitising systems (photosensitisers allied to an appropriate light source) against a range of both Gram-negative and Gram-positive bacteria. However, a large proportion of this body of work has concentrated its efforts on the photosensitisation of planktonic organisms. In the case of oral infections, specifically periodontal diseases, it is known that the putative aetiological agents are present *in vivo* as biofilms. Hence, to fully gauge the potential of a photosensitising system for the treatment of periodontal diseases it is essential to assess the efficaciousness of the chosen photosensitiser and allied light source against biofilms.

The initial aim of this investigation was to identify a suitable photosensitiser for use in conjunction with a HeNe laser light source. Several photosensitisers were chosen for initial screening, many of which had previously been employed as photosensitising compounds against both Gram-positive and Gram-negative organisms. Based on an initial screening, which included an assay to measure singlet oxygen production and the lethal photosensitisation of *S. sanguinis*, TBO was chosen. The two grades of TBO available for the study were then subjected to a broader examination, to determine their efficacy against a range of oral bacteria that included several putative periodontal pathogens; this revealed no

observable difference in efficacy between the two grades of TBO against planktonic oral organisms. Building upon this, the next aim was to determine whether TBO in conjunction with HeNe laser light could effectively photosensitise bacteria when they were present in biofilms. Rudimentary mono-species biofilms were grown on membrane filters, both commensal and pathogenic organisms were used to form biofilms; no appreciable difference in efficacy was apparent between the two grades of TBO, however it was clear that merely cultivating a single species as a biofilm increased appreciably its resistance to lethal photosensitisation. The filter membrane methodology was subsequently employed to enable the rapid formation of multi-species biofilms from an inoculum composed principally of pooled human saliva. Again, the recalcitrance of the biofilm phenotype was apparent and, as before, no difference in efficacy between the two grades of TBO could be detected. The filter membrane method of cultivating biofilms was used initially due to the rapidity with which biofilms could be generated.

The next logical evolutionary step was to develop a laboratory model that was capable of producing large numbers of biofilms that mimicked, as regards composition and structure, subgingival plaques found *in vivo*; thus permitting an assessment of PDT as a potential treatment for periodontal diseases. Before biofilms cultivated in a constant depth film fermentor (CDFF) were subjected to lethal photosensitisation, a thorough analysis of the biofilms generated within the model system was performed. What was essential to establish was that the

biofilms produced within the CDFF could be considered an approximation of what one could reasonably expect to find within the confines of a periodontal lesion *in vivo*. The microbial community generated within the CDFF was cultivated from an inoculum consisting of pooled sub-gingival plaque taken from periodontal disease lesions. The CDFF-grown biofilms were sampled periodically (24 h, 72 h, 168 h, 240 h and 336 h), the particular sampling points were chosen to allow for each stage in the development of the biofilms to be analysed using a polyphasic approach. The polyphasic approach incorporated a culture independent based molecular technique, DGGE, and the culture-dependent sequencing of the microbiota sampled at each time point, additionally community level physiological profiling (CLPP) was used to quantify the metabolic diversity inherent within the biofilms. These quantitative data were supplemented with microscopic data that were provided using CLSM as well as SEM and TEM.

Interestingly, there were commonalities over the course of both fermentor runs that were evident when results were compared. A pertinent example is found if one compares the number of bacterial taxa isolated and sequenced at each sampling point and the number of CLPP positives recorded at each sampling point, specifically after a 6 h incubation period. Both sets of results display the same distinctive 'fingerprint', both reached their zenith as regards the number of taxa identified and CLPP positives recorded at the168 h sampling point. Indeed, during the course of both runs, the number of taxa identified at each sampling point increased to reach its maximum at the 168 h sampling point, thereafter

declining gradually at the last two sampling points. This trend mirrored exactly what was found when the CLPP data were analysed, this showed the number of CLPP positives increased up to the 168 h sampling point and decreased gradually through the final two sampling points. Another example of concordance between results occured if one examines the viable count data for both runs and the visual archive of biofilm maturation provided by confocal microscopy. What was evident over the course of both runs was that the viable count data, specifically the total anaerobic count, increased over successive sampling time-points to the 168 h sampling point, whereupon it then gradually decreased over the remaining two sampling points. This trend was identifiable in both runs. Interestingly, this mirrored what was seen via the confocal microscopy, whereby up to the 168 h sampling point the biofilms were composed principally of viable cells, subsequently over the course of the final two sampling points, the ratio of non-viable to viable cells increased, such that at the final sampling point the biofilms seemed to be composed principally of non-viable cells. Indeed, the increase in species diversity over the course of both runs as revealed by the sequencing of the cultivable microbiota concurs with the visual images provided by both SEM and TEM. The electron microscopy showed what appeared to be highly homogenous biofilms initially, diversifying at each successive sampling point such that a wide range of bacterial morphotypes were evident at both the 240 h and 336 h sampling points. The degree of concurrence displayed when the results are analysed in their entirety is reassuring and vindicates the use of a polyphasic approach; this multi-faceted methodology

permitted the scrutiny of all facets of biofilm development within the CDFF. The biofilms generated within the CDFF demonstrated increased complexity as a function of time as regards species composition, metabolic plasticity and biofilm architecture. Thus the polyphasic approach allows one to tentatively conclude that the biofilms cultivated within the CDFF do represent an acceptable approximation of what one may reasonably expect to find within a periodontal lesion *in vivo*.

Upon completion of the analysis of the biofilms generated within the CDFF, which established that the cultivated biofilms do constitute an acceptable approximation of a periodontal disease lesion, the next aim was to determine their susceptibility to lethal photosensitisation. The results of the lethal photosensitisation studies were rather interesting; this demonstrated that CDFF-grown biofilms were indeed susceptible to lethal photosensitisation when using TBO in conjunction with HeNe laser light. Indeed, if one compares the results obtained in chapters 4 and 5, the lethal photosensitisation of mono- and multi-species species biofilms cultivated on membrane filters and the results obtained in chapter 9 where CDFF cultivated multi-species biofilms were subjected to lethal photosensitisation, there does not appear to be an appreciable difference in the kill. In nearly all instances, the kills obtained were of the order of between 1 $\log_{10}$ and 2 $\log_{10}$, a 90-99% reduction in the number of recovered viable bacteria. The corollary being perhaps that, regardless of composition and method of cultivation, the

biofilm phenotype confers decreased susceptibility to lethal photosensitisation when using TBO in conjunction with HeNe laser light.

The final aim of the investigation was a comparative analysis of the efficaciousness of lethal photosensitisation when compared to a commonly prescribed anti-microbial compound, chlorhexidine digluconate. The 60 s versus 60 s comparison was carried out primarily as this is approximately the amount of time a dentist could reasonably spend when treating a single periodontal pocket. The results demonstrated that, under broadly comparable experimental conditions, there does not appear to be an appreciable difference in efficacy, suggesting that lethal photosensitisation, under the experimental conditions employed, brings to bear a comparable anti-microbial effect as 0.2 % chlorhexidine digluconate. However, one must bear in mind that the experimental comparison of exposure to chlorhexidine digluconate with lethal photosensitisation was somewhat simplistic. As alluded to previously the level of kill induced by lethal photosensitisation is dependent upon a number of variables including the light energy dose delivered and the concentration of photosensitiser applied. Throughout the course of the study the only laser available for use delivered a fixed power output; if a higher power laser had been available this may have resulted in much greater kills. Indeed the study was rather limited due to the power output of the laser available; this could not be easily remedied as increasing the exposure time to compensate may have resulted in the decreased

viability of the bacteria composing the biofilms, due to the known fastidious nature of many putative periodontopathogens.

The results of this study have demonstrated that both pathogenic and commensal oral bacteria are indeed susceptible to the antimicrobial action exerted by TBO when allied to a HeNe laser. The degree of susceptibility varied depending on the organism being photosensitised; planktonic oral organisms were shown to be highly susceptible to the photodynamic action of TBO, however this did vary appreciably depending on the nature of the cell wall of the organism being sensitised. In contrast, biofilms proved to be comparatively recalcitrant to lethal photosensitisation. The filter membrane methodology was used as a rapid way to generate both mono and multi-species biofilms. The CDFF was shown to be capable of generating large numbers of biofilms that were an approximation of what one may expect to find within a periodontal lesion. Finally, the fermentor-grown biofilms were subjected to lethal photosensitisation using TBO and HeNe laser light, appreciable kills were recorded. Indeed lethal photosensitisation was found to exert a comparable anti-microbial effect to 0.2 % chlorhexidine digluconate, suggesting that lethal photosensitisation does appear to be, at least in principal, a viable alternative to traditional adjunctive treatments that are routinely administered for the treatment of periodontitis.

REFERENCES

Absolom DR, Lamberti FV, Policova Z, Zingg W, Van Oss CJ, Neumann AW (1983). Surface thermodynamics of bacterial adhesion. Applied and Environmental Microbiology **46**:90-97

Ackroyd R, Kelty C, Brown N, Reed M (2001). The history of photodetection and photodynamic therapy. Photochemistry and photobiology **74**:656-669

Aldsworth TG, MacFarlane TW (2001). A novel in vitro system to grow films of oral bacteria for the study of human root surface caries. Journal of Applied Microbiology **91**:139-146.

Altschul SF, Madden TL, Schaffer AA et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acid Results **25**:3389-3402

Anderl JN, Franklin MJ, Stewart PS (2000). Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. Antibacterial Agents and Chemotherapy **44**: 1818-1824

Anderson SA, Sissons CH, Coleman MJ, Wong L (2002). Application of carbon source utilisation patterns to measure the metabolic similarity of complex dental plaque biofilm microcosms. Applied and Environmental Microbiology **68**:5779-5783

Archibald LK, Gaynes RP (1997). Hospital acquired infections in the United States: the importance of inter-hospital comparisons. Noscomial Infection **11**:245-255

Barber PM (1995). The analysis of dental plaque sections. Microbial Ecology in Health and disease **8**:309-311

Bhatti M, MacRobert A, Meghji S, Henderson B, Wilson M (1998). A study of the uptake of Toluidine Blue O by *Porphyromonas gingivalis* and the mechanism of lethal photosensitisation. Photochemistry and photobiology. **68**: 370-376

Bhatti M, Nair SP, MacRobert AJ, Henderson B, Shepherd P, Cridland J, Wilson M (2001). Identification of photolabile outer membrane proteins of *Porphyromonas gingivalis*. Current Microbiology **43**:96-99

Bickel M, Cimasoni G, Andersen E (1985). Flow and albumin content of early (pre-inflammatory) gingival crevicular fluid from human subjects. Archives of Oral Biology **30**:599-602

Bigger J (1944). Treatment of Staphylococcal infectons with penicillin by intermittent sterilisation. The lancet **ii**:497-501

Bloomquist CG, Reilly BE, Liljemark WF (1996). Adherence, accumulation and cell division of a natural adherent bacterial population. Journal of Bacteriology **178**: 1172-1177

Boon N, Marle C, Top EM, Verstraete W (2000). Comparison of the spatial homogeneity of physico-chemical parameters and bacterial 16s rRNA genes in sediment samples from a dumping site for dredging sludge. Applied Microbiology and Biotechnology **53**:742-747

Bos R, Van der Mei HC, Busscher HJ (1999.) Physico-chemistry of initial adherence interactions its mechanisms and methods for study. FEMS Microbiology Reviews **23**:179-230

Bostrom L, Linder LE, Bergstrom J (1998). Clinical expression of TNF-alpha in smoking associated periodontal disease. Journal of Clinical Periodontology **25**:767-773

Bradshaw DJ (1995). Metabolic responses in biofilms. Microbial Ecology in Health and Disease **8**:313-316

Bradshaw DJ, Marsh PD, Allison C, Schilling KM (1996). Effect of oxygen, inoculum composition and flow rate on development of mixed-culture oral biofilms. Microbiology **142**:623-629

Bramanti TE, Wong GG, Weintraub ST, Holt SC (1989). Chemical characterization and biologic properties of lipopolysaccharide from *Bacteroides gingivalis* W50, W83 and ATCC 33277. Oral Microbiology and Immunology **4**:183-192

Brecx M, Ronstrom A, Theilade J, Attstrom R (1981). Early formation of dental plaque on plastic films. 2. Electron microscopic observations. Journal of Periodontal Research **16**:213-227

Brooun A, Liu S, Lewis K (2000). A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. Antimicrobial Agents and Chemotherapy **44**:640-646

Brown MRW, Williams P (1985). Influence of substrate limitation and growth phase on sensitivity to antimicrobial agents. Journal of Antimicrobial Chemotherapy **15**(Suppl. A):7-14

Brown MRW, Allison DG, Gilbert P (1988). Resistance of bacterial biofilms to antibiotics: a growth-related effect? Journal of Antimicrobial Chemotherapy **22**:777-80.

Brown LJ, Loe H (1993). Prevalence, extent, severity and progression of periodontal disease. Periodontology 2000. **2**:57-71

Burch PE (1989). DNA damage and cell lethality by photodynamically produced oxygen radicals. Ph.D. Thesis, Rice university, USA

Burns T, Wilson M, Pearson GJ (1996). Mechanism of killing *Streptococcus mutans*. Proc Soc Instr Eng (SPIE) **2625**:288-297

Busscher HJ, Doornbusch GI, Van Der Mei HC (1992). Adhesion of mutans streptococci to glass with and without a salivary coating as studied in a parallel plate flow chamber. Journal of Dental Results **71**:491-500

Busscher HJ, Geertseema-Doornbusch GI, Van der Mei HC (1993). On mechanisms of oral microbial adhesion. Journal of Applied Bacteriology symposium supplement **74**:136s-142s

Campbell CD, Grayston SJ, Hirst DJ (1997). Use of rhizosphere carbon sources in sole carbon source tests to discriminate soil microbial communities. Journal of Microbiological Methods **30**:33-41

Carlsson J (1997). Bacterial metabolism in dental biofilms. Advances in Dental Research **11**:75-80

Carruth J (1998). Clinical applications of photodynamic therapy. International Journal of Clinical Practice **52**:39-42

Cauvin JF (1815). Des bienfaits de l'insolation. Ph.D. Thesis, University of Paris, France.

Christersson LA, Zambon JJ, Genco RJ (1991). Dental bacterial plaques nature and role in periodontal disease. Journal of Clinical Periodontology **18**: 441-446

Christersson LA, Norderyd OM, Puchalsky CS (1993). Topical application of tetracycline-HCl in human periodontitis. Journal of Clinical Periodontology **20**:88-95

Ciantar M (1995). Chemical agents in periodontal therapy: the use or misuse? Dental Update **22**:238-242

Ciancio SG, Mather ML, McMullen JA (1980). An evaluation of minocycline in patients with periodontal disease. Journal of Periodontology **51**: 530-534

Cimasoni G (1983). Crevicular fluid updated. Monographs in Oral Science Vol.12, Karger Press, Basel pp70-71

Cochran WC, Mc Feters GA, Stewart PS (2000). Reduced susceptibility of thin *Pseudomonas aeruginosa* biofilms to hydrogen peroxide and monochloramine. Journal of Applied Microbiology **88**:22-30

Cohan FM (2002). What are bacterial species. Annual Review of Microbiology **56**:457-487

Coquet L, Junter GA, Jouenne T (1998). Resistance of artificial biofilms of *Pseudomonas aeruginosa* to imipenem and tobramycin. Journal of Antimicrobial Chemotherapy **42**:755-760

Costerton JW (1995). Overview of microbial biofilms. Journal of Industrial Microbiology **15**:137-140

Costerton JW (1999a). Discussion: Introduction to biofilm. International journal of Antimicrobial Agents **11**:217-221

Costerton JW, Stewart PS, Greenberg EP (1999b). Bacterial biofilms: a common cause of persistent infections. Science **284**:1318-1322

Cummins D, Moss MC, Jones CL, Howard CV, Cummins PG (1992). Confocal microscopy of dental plaque. Binary **4**:86-91

Curtis MA, Griffiths GS, Price SJ, Coulthurst SK, Johnson NW (1988). The total protein concentration of gingival crevicular fluid. Variation with sampling time and gingival inflammation. Journal of Clinical Periodontology **15**:628-632

Darveau RP, Tanner A, Page RC (1997). The microbial challenge in periodontitis. Periodontology 2000 **14**:12-32

Darzynkiewicz Z, Carter PS (1988). Photosensitizing effects of the tricyclic heteroaromatic cationic dyes Pyronin Y and Toluidine Blue O. Cancer research **48**:1295-1299.

De Beer D, Stoodley P, Roe F, Lewandowski Z (1994) Effects of biofilm structures on oxygen distribution and mass transport. Biotechnology and Bioengineering **43**:1131-1138

Demuth DR, Irvine DC, Costerton JW, Cook GS, Lamont RJ (2001). Discrete protein determinant directs the species-specific adherence of *Porphyromonas gingivalis* to oral streptococci. Infection and Immunity **69**:5736-5741

Deretic V, Dikshit R, Konyecsni WM, Chakranbarty AM, Misra TK (1989). The algR gene, which regulates mucoidy in *Pseudomonas aeruginosa*, belongs to a class of environmentally responsive genes. Journal of Bacteriology **171**:1278-1283

Diaz PI, Zilm PS, Rogers AH (2002). *Fusobacterium nucleatum* supports the growth of *Porphyromonas gingivalis* in oxygenated and carbon dioxide depleted environments. Microbiology **148**:467-472

Dibdin G, Wimpenny J (1999). Steady-state biofilm: Practical and theoretical models. Methods in Enzymology **310**:296-322

Dobson J, Wison M (1992). Sensitization of oral bacteria in biofilms to killing by light from a low-power laser. Archives of Oral Biology **37**:883-887

Engelen B, Meinken K, von Wintzingerode F, Heuer H, Malkomes HP, Backhaus H (1998). Monitoring impact of a pesticide treatment on bacterial soil communities by metabolic and genetic fingerprinting in addition to conventional testing procedures. Applied and Environmental Microbiology **64**: 2814-2821

Epstein, JM (1990). Phototherapy and photochemotherapy. New England Journal of Medicine **32**:1149-1151

Estreicher A, Broggiato A, Duroux P, Andersen E, Cimasoni G (1996). Low molecular weight proteins in human gingival crevicular fluid. Archives of Oral Biology **41**:733-738

Finsen NR (1901). Phototherapy. Edward Arnold, London

Fischer F, Graschew G, Sinn HJ, Maier-Borst W, Lorenz WJ, Schlag PM (1998). A chemical dosimeter for the determination of the photodynamic activity of photosensitisers. Clinica Chimica Acta **274**:89-104

Frandsen EVG (1994). Carbohydrate depletion of immunoglobulin A1 by oral species of Gram-positive rods. Oral Microbiology and Immunology **9**:352-358

Garland JL Mills AL (1991). Classification and characterisation of heterotrophic microbial communities on the basis of patterns of community-level-sole-carbon-source-utilization. Applied and Environmental Microbiology **57**:2351-2359

Garland JL (1999). Potential and limitations of BIOLOG for microbial community analysis. In Proceedings of the 8th International Symposium on Microbial Ecology. Bell CR, Brylinsky M, Johnson-Green M (eds). Atlantic Canada Society for Microbial Ecology

Genco RJ, Goldman HM, Cotten DW (eds) (1990). Contemporary periodontics. C.V Mosby company, St. Louis, MO, U.S.A

Gibbons RJ, Nygaard M (1970). Interbacterial aggregation of plaque bacteria. Archives of Oral Biology **15**:1397-1400

Gibbons RJ (1996). Role of adhesion in microbial colonisation of host tissues: A contribution to oral microbiology. Journal of Dental Results **75**:866-870

Gilbert P, Das J, Foley I (1997). Biofilm susceptibility to antimicrobials. Advances in Dental Research. **11**:160-167

Gilbert P, Allison DG, McBain AJ (2002). Biofilms *in vitro* and *in vivo*: do singular mechanisms imply cross-resistance? Journal of Applied Microbiology Symposium Supplement **92**:98S-110S

Greenstein G, Caton J (1990). Periodontal disease activity: A critical assessment. Journal of Periodontology **61**:543-552

Greenstein G, Lamster I (2000). Changing periodontal paradigms: Therapeutic implications. The International Journal of Periodontics and Restorative Dentistry **20**: 337-357

Griffiths MA, Wren BW, Wilson M (1997). Killing methicillin resitant *Staphylococcus aureus* in vitro using aluminium disulphonated phthalocyanine, a light-activated antimicrobial agent. Journal of Antimicrobial Chemotherapy **40**:873-876

Haack SK, Garchow H, Klug MJ, Forney LJ (1995). Analysis of factors affecting the accuracy, reproducibility and interpretation of microbial community carbon source utilisation patterns. Applied and Environmental Microbiology **61**:1458-1468

Hamblin MR, O'Donnell DA, Murthy N, Rajagopalan K, Michaud N, Sherwood ME, Hasan T (2002). Polycationic photosensitiser conjugates: effects of chain length and Gram classification on the photodynamic inactivation of bacteria. Journal of Antimicrobial Chemotherapy **49**:941-951.

Hamilton WA, Characklis WG (1989). Relative activities of cells in suspension and in biofilms, Characklis WG, Wilderer PA (eds).Structure and function of Biofilms. John Wiley and sons Ltd, pp199-219

Hardie M (1992). Oral microbiology: current concepts in the microbiology of dental caries and periodontal disease. British Dental Journal **172**:271-278

Havemose-Poulsen A, Holmstrup P (1997). Factors affecting IL-1-mediated collagen metabolism by fibroblasts and the pathogenesis of periodontal disease: a review of the literature. Critical Reviews in Oral Biology and Medicine **8**:217-236

Heuer H, Smalla K (1997). Evaluation of community-level catabolic profiling using biolog GN microplates to study microbial community changes in potato phyllosphere. Journal of Microbiological Methods **30**:49-61

Hoag PM, Pawlak EA. (1990) Essentials of Periodontics. C.V Mosby company. St. Louis, MO, U.S.A, pp.181

Holt SC, Bramanti TE (1991). Factors in virulence expression and their role in periodontal disease pathogensis. Critical Reviews in Oral Biology and Medicine **2**:177-281

Homer KA, Beighton D (1992). Synergistic degradation of bovine serum albumin by mutans streptococci and other dental plaque bacteria. FEMS Microbiology Letters **90**:259-262

Hope CK, Clements D, Wilson M (2002). Determining the spatial distribution of viable and nonviable bacteria in hydrated microcosm dental plaques by viability profiling. Journal of Applied Microbiology **93**:448-455.

Hope CK, Wilson M (2004). Analysis of the effects of chlorhexidine on oral biofilm vitality and structure based on viability profiling and an indicator of membrane integrity. Antimicrobial Agents and Chemotherapy **48**:1461-1468

Ito T (1977a). Toluidine blue: The mode of photodynamic action in yeast cells. Photochemistry and Photobiology **25**:47-53

Ito T, Kobayashi K (1977b). *In vivo* evidence for the photodynamic membrane damage as a determining step for the inactivation of yeast cells sensitized by toluidine blue. Photochemistry and Photobiology **25**:399-401

Jenkins WMM, Allan CJ (1994) Guide to periodontics. Butterworth-Heinemann, Oxford, England, pp1-24

Jenkins WMM, Allan CJ (1999) Periodontics: a synopsis. Butterworth-Heinemann, Oxford, England, pp.66

Jones HC, Roth IL, Sanders WM (1969). Electron Microscopic study of a slime layer. Journal of Bacteriology **99**:316-325

Jones SJ (1972). A special relationship between spherical and filamentous microorganisms in mature dental plaque. Archives of Oral Biology **17**:613-616

Jorgensen MG, Slots J (2001). Antimicrobials in periodontal maintenance. The Journal of Dental Hygenie **75**:233-239

Kaeberlein T, Lweis K, Epstein SS (2002). Isolating 'uncultivable' microorganisms in pure culture in a simulated natural environment. Science **296**:1127-1129

Kamma JJ, Diamanti-Kipoti A, Nakou M, Mitsis F (2000). Profile of subgingival microbiota in children with mixed dentition. Oral Microbiology and Immunology **15**:103-111.

Kilian M (1995). Topical antibiotics and gastric flora. British Dental Journal **178**:94

Kinane DF, Radvar M (1999). A six-month comparison of three periodontal local antimicrobial therapies in persistent periodontal pockets. Journal of Periodontology **70**:1-7

Kinane DF (2001). Periodontal disease in children and adolescents: introduction and classification. Periodontology 2000 **26**:7-15

Kolenbrander PE, London J (1993). Adhere today, here tomorrow: Oral bacterial adherence. Journal of Bacteriology **175**:3247-3252

Kornman KS, Page RC, Tonetti MS (1997). The host response to the microbial challenge in periodontitis: Assembling the players. Periodontology 2000 **14**:33-53

Kresse AU, Beltrametti F, Muller A, Ebel F, Guzman CA (2000). Characterization of Sep L of enterohemorrhagic *Escherichia coli.* Journal of Bacteriology **182**:6490-6498

Kuyyakanond T, Quesnel LB (1992). The mechanism of action of chlorhexidine. FEMS Microbiology Letters **100**:211-215

Kulik EM, Sandmeier H, Hinni K, Meyer J (2001). Identification of archaeal rDNA from subgingival dental plaque by PCR amplification and sequence analysis. FEMS Microbiology Letters **9818**:1-5

Lamont RJ, Jenkinson HF (1998). Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis.* Microbiology and MolecularBiology Reviews **62**:1244-1263

Lamont RJ, El-Sabeany A, Park Y, Cook GS, Costerton JW, Demuth DR (2002). Role of the Streptcoccus gordonii Sspb protein in the development of *Porphyromonas gingivalis* biofilms on streptococcal surfaces. Microbiology **148**:1627-1636

Lane DJ (1991) 16s/23s rRNA sequencing, In E.Stackebrandt and M. Goodfelllow (eds), Nucleic acid techniques in bacterial systematics. John Wiley and Sons Ltd., West Sussex, U.K, pp.115-175

Laine MM, Haario H, Jorgensen KS (1997). Microbial functional activity during composting of chlorophenol – contaminated sawmill soil. Journal of Microbiological Methods **30**:21-32

La Tourette Prosser B, Taylor D, Dix BA, Cleeland R (1987). Method of evaluating effects of antibiotics on bacterial bofilm. Antimicrobial Agents and Chemotherapy **31**:1502-1506

Lawlor K, Knight BP, Barbosa-Jefferson VL, Lane PW, Lilley AK, Paton GI, McGrath SP, O'Flaherty SM, Hirsch PR (2000). Comparison of methods to investigate microbial populations in soils under different agricultural management. FEMS Microbiology Ecology **33**:129-137

Lawrence JR, Wolfaart GM, Korber DR (1993). Diffusion of size fractionated dextrans in biofilm matrices by confocal scanning laser microscopy. (abstract) Canadian society for Microbiology Annual Meeting, Toronto

Lewis K (2001). Riddle of biofilm resistance. Antimicrobial Agents and Chemotherapy **45**: 999-1007

Lindemann RA, Economou JS, Rothermel H (1988). Production of interleukin-1 and tumour necrosis factor by human peripheral monocytes activated by periodontal bacteria and extracted liposccharides. Journal of Dental Results **67**:1131-1135

Listgarten MA, Mayo H, Amsterdam M (1973). Ultrastructure of the attachment device between coccal and filamentous micro-organisms in 'corn cob' formations of dental plaque. Archives of Oral Biology **18**:651-656

Listgarten MA, Mayo HE, Tremblay R (1975). Development of dental plaque on epoxy resin crowns in man. Journal of Periodontology **46**:10-26

Listgarten MA (1976). Structure of the microbial flora associated with periodontal health and disease in man. A light and electron microscopic study. Journal of Periodontology **47**:1-18

Loe H, Theilade E, Jensen SB (1965). Experimental gingivitis in man. Journal of Periodontology **36**:177-187

Loesche WJ (1976). Chemotherapy of dental plaque infections. Oral Sciences Review **9**:63-107

Loesche WJ, Gusberti F, Mettraux G, Higgins T, Syed S (1983). Relationship between oxygen tension and subgingival bacterial pockets in untreated human periodontal pockets. Infection and Immunity **42**:659-667

Loesche WJ (1986). Role of *Streptococus mutans* in human dental decay. Microbiology Reviews **50**:353-380

Loesche WJ, Lopatin DE, Stoll J, Van Poperin N, Hujoel PP (1992). Comparison of various detection methods for periodontopathogenic bacteria: can culture be considered the primary reference standard? Journal of Clinical Microbiology **30**:418-426

Mah TFC, O'Toole GA (2001). Mechanisms of biofilm resistance to antimicrobial agents. Trends in Microbiology **9**:34-39

Maidak BL, Cole JR, Lilburn TG et al.(2000). The RDP (Ribosomal Database Project) continues. Nucleic Acids Results **28**:173-174

Maira-Litran T, Allison DG, Gilbert P (2000). Expression of the multiple antibiotic resistance operon (mar) during growth of *Escherichia coli* as a biofilm. Journal of Applied Microbiology **88**:243-247

Malik Z, Hanania J, Nitzan Y (1990). New trends in photobiology bactericidal effects of photoactivated porphyrins – an alternative approach to antimicrobial drugs. Journal of Photochemistry and Photobiology B: Biology **5**:281-293.

Malik Z, Landan H, Nitzan Y (1992). Photodynamic inactivation of Gram-negative bacteria: problems and possible solutions. Journal of Photochemistry and Photobiology B **14**:262-266

Marquis RE (1968). Salt-induced contraction of bacterial cell walls. Journal of Bacteriology **95**:775-781

Marsh P, Martin M. (1992) Oral Microbiology. Chapman and Hall, London, U.K

Marsh PD (1994). Microbial ecology of dental plaque and its significance in health and disease. Advances in Dental Research **8**:263-271

Marsh PD (1999). Microbiologic aspects of dental plaque and dental caries. Dent Clin North Am. **43**: 599-614

Marsh PD (2003). Are dental diseases examples of ecological catastrophes. Microbiology **149**:279-294.

Martin JP, Logsdon N (1987a). The role of oxygen radicals in dye-mediated photodynamic effects in *Escherichia coli* B. The Journal of Biological Chemistry **282**:7213-7219.

Martin JP, Logsdon M (1987b). Oxygen radicals are generated by dye-mediated intracellular photooxidants: a role for superoxide in photodynamic effects. Archives of Biochemistry and Biophysics **256**:39-49.

Martinetto P, Gariglio M, Lombard GF, Fiscella B, Boggio F (1986). Bactericidal effects induced by laser irradiation and haematoporphyrin against Gram-positive and Gram-negative microorganisms. Drugs Exptl. Clin. Res. **XII**:335-342.

Martinez-Canut P, Lorca A, Magan R (1996). Smoking and periodontal disease severity. Journal of Clinical Periodontology **22**:743-749

Massol-Deya AA, Whallon J, Hickey RF, Tiedge JM (1995). Channel structures in aerobic biofilms of fixed-film reactors treating contaminated groundwater. Applied and Environmental Microbiology **61**:769-777

McBain AJ, Bartolo RG, Catrenich CE, Charbonneau D, Ledder RG, Gilbert P (2003). Growth and molecular characterisation of dental plaque microcosms. Journal of Applied Microbiology **94**:655-664

McCoy WF, Bryers JD, Robbins J, Costerton JW (1981). Observations of fouling biofilm formation. Canadian Journal of Microbiology **27**:910-917

Mc Inroy SG, Campbell CD, Haukka KE, Odee DW, Sprent JI, Wang WJ, Young PW, Sutherland JM (1999). Characterisation of rhizobia from African acaias and other tropical woody legumes using Biolog and partial 16s rRNA sequencing. FEMS Microbiology Letters **170**:111-117

Meghji S, Henderson B, Nair S, Wilson M (1992). Inhibition of bone DNA and collagen production by surface-associated material from bacteria implicated in the pathology of periodontal disease. Journal of periodontology **63**:736-742

Merchat M, Bertolini G, Giacomini P, Villanueva A, Jori G (1996). Meso-substituted cationic porpyrins as efficient photosensitizers of Gram-positive and Gram-negative bacteria. Journal of Photochemistry and Photobiology B: Biology **32**:153-157.

Mettraux GR, Gusberti FA, Graf H (1984). Oxygen tension (pO_2) in untreated periodontal pockets. Journal of Periodontology **55**:516-521

Miambi E, Guyot JP, Ampe F (2003). Identification, isolation and quantification of representative bacteria from fermented cassava dough using an integrated approach of culture-dependent and culture-independent methods. International Journal of Food Microbiology **82**:111-120

Millson CE, Wilson M, MacRobert AJ, Bedwell J, Bown SG (1996). The killing of *Helicobacter pylori* by low-power laser light in the presence of a photosensitiser. Journal of Medical Microbiology **44**:245-252

Minnock A, Vernon DI, Schofield J, Griffiths J, Parish H, Brown SB (1996). Photoinactivation of bacteria. Use of a cationic water-soluble zinc phthalocyanine to photoinactivate both Gram-negative and Gram-positive bacteria. Journal of Photochemistry and Photobiology B: Biology **32**:159-164.

Mohr H, Bachmann B, Klein-Struckmeier A, Lambrecht B (1997). Virus inactivation of blood products by phenothiazine dyes and light. Photochemistry and Photobiology **65**:441-445

Mombelli A, Samaranayake LP (2004). Topical and systemic antibiotics in the management of periodontal diseases. International Dental Journal **54**:3-14

Moore WEC, Holdeman LV, Smibert RM, Hash DE, Burmeister JA, Ranney RR (1982). Bacteriology of severe periodontitis in young adult humans. Infection and Immunity **38**:1137-1148

Moore WEC, Holdeman LV, Cato EP, Smibert RM, Burmeister JA, Raney RR (1983). Bacteriology of moderate (chronic) periodontitis in mature adult humans. Infection and Immunity **42**:510-515

Moore WEC, Moore LVH (1994). The bacteria of periodontal diseases. Periodontology 2000 **5**:66-77

Muller M, Lindmark DG, McLaughlin J (1977). Mode of action of metronidazole on anaerobic microorganisms. In: Finegold S ed. Proceeedings of the International Metronidazole Conference, Montreal, Quebec, Canada, 26-28 May 1976. Amsterdam: Excerpta Medica pp:12-19

Muyzer G, Dewaal EC, Uitterlinden AG (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16s rDNA fragments. Applied and Environmental Microbiology **59**:695-700

Muyzer G, Smalla K (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek **73**:127-141
Muyzer G (1999). DGGE/TGGE a method for identifying genes from natural ecosystems. Current Opinion in Microbiology **2**:317-322

Nakagawa T, Saito A, Hosaka Y, Yamada S, Tsunoda M, Sato T, Ishikawa T (1990). Bactericidal effects on subgingival bacteria of irrigation with a povidone-iodine solution (Neo-jodin). Bull Tokyo Dent Coll **31**:199-203

Netuschil L, Reich E, Unteregger G, Sculean A, Brecx M (1998). A pilot study of confocal laser scanning microscopy for the assessment of undisturbed dental plaque vitality and topography. Archives of Oral Biololgy **43**:277-285.

Neu TR, Marshall KC (1990). Bacterial polymers: physico-chemical aspects of their interactions at interfaces. J. Biomaterials Appl. **5**:107-133

Nikaido H (1988). Bacterial resistance to antibiotics as a function of outer membrane permeability. Journal of Antimicrobial Chemotherapy **22**(suppl. A): 17-22

Nitzan Y, Schainberg B, Malik Z (1989). The mechanism of photodynamic inactivation of *staphylococcus aureus* by deuteroprophyrin. Current Microbiology **19**:265-269.

Nitzan Y, Gutterman M, Malik Z, Ehrenberg B (1992). Inactivation of Gram-negative bacteria by photosensitized porphyrins. Photochemistry and Photobiology **55**:89-96.

Nitzan Y, Balzam-Sudakevitz A, Ashkenazi H (1998). Eradication of *Acinetobacter baumannii* by photosensitized agents *in vitro*. Journal of Photochemistry and Photobiology B: Biology **42**:211-218.

Norling T, Lading P, Engstrom S, Larsson K, Krog N, Nisen SS (1992). Formulation of a drug delivery system based on a mixture of monoglycerides and triglycerides for use in the treatment of periodontal disease. Journal of Clinical Periodontology **19**:687-692

Novak MJ, Cohen HJ (1991). Depolarization of polymorphonuclear leukocytes by *Porphyromonas* (Bacteroides) *gingivalis* 381 in the absence of respiratory burst activation. Infection and Immunity **59**:3134-3142

Nyvad B, Kilian M (1987a). Microbiology of the early colonisation of human enamel and root surfaces *in vivo*. Scandinavian Journal of Dental Results **95**:369-380

Nyvad B, Fejerskov O (1987b). Scanning electron microscopy of early microbial colonisation of human enamel and root surfaces *in vivo*. Scandinavian Journal of Dental Results **95**:287-296

Nyvad B, Fejerskov O (1987c).Transmission electron microscopy of early microbial colonisation of human enamel and root surfaces *in vivo*. Scandinavian Journal of Dental Results **95**:297-307

O'Connell S, Lawson RD, Watwood ME, Lehman RM (2000). Basic program for reduction of data from communitiy-level physiological profiling using Biolog microplates: rationale and critical interpretation of data. Journal of Microbiological Methods **40**:213-220

O'Connor BC, Newman HN, Wilson M (1990). Susceptibility and resistance of plaque bacteria to minocycline. Journal of Periodontology. **61**:228-233

Ogino A, Koshikawa H, Nakahara T, Uchiyama H (2001). Succession of microbial communities during a biostimulation process as evaluated by DGGE and clone library analyses. Journal of Applied Microbiology **91**:625-635

Okabe S, Santegoeds CM, Watanabe Y, De Beer D (2002). Successional development of sulphate-reducing bacterial populatons and their activities in an activated sludge immobilised agar gel film. Biotechnology and Bioengineering **78**:119-130

Palmer RJ, Kazmerzak K, Hansen MC, Kolenbrander PE (2001). Mutalism versus independence: stratergies of mixed-species oral biofilms *in vitro* using saliva as the sole nutrient source. Infection and Immunity **69**:5794-5804

Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, Dewhirst FE (2001.) Bacterial diversity in human subgingival plaque. Journal of Bacteriology **183**:3370-3783

Peters AC, Wimpenny JW (1988). A constant-depth laboratory model film fermentor. Biotechnology and Bioengineering **32**:263-270

Phillips D (1997). Chemical mechanisms in photodynamic therapy with phthalocynanines. Progress in Reaction Kinetics **22**:189-192

Pine, CM, Pitts, NB, Steele, JG, Nunn, JN and Treasure, E (2001). Dental restorations in adults in the UK in 1998 and implications for the future. British Dental Journal **190**:4-8

Poyart C, Quesne G, Coulon S, Berche P, Trieu-cuot P (1998). Identification of Streptococci to species level by sequencing the gene encoding the manganese-dependent superoxide dismutase. Journal of Clinical Microbiology **36**:41-47

Poolman B (1993). Energy transduction in lactic acid bacteria. FEMS Microbiology Reviews **12**:125-148

Pratten J, Smith AW, Wilson M (1998a). Response of single species biofilms and microcosm dental plaques to pulsing with chlorhexidine. Journal of Antimicrobial Chemotherapy **42**:453-459

Pratten J, Barnett P, Wilson M (1998b). Composition and susceptibilty to chlorhexidine of multispecies biofilms of oral bacteria. Applied and Environmental Microbiology **64**:3515-3519

Pratten J, Wilson M (1999). Antimicrobial susceptibility and composition of microcosm dental plaques supplemented with sucrose. Antimicrobial Agents and Chemotherapy **43**:1595-1599

Pratten J, Andrews CS, Craig DQM, Wilson M (2000a). Structural studies of microcosm dental plaques grown under different nutritional conditions. FEMS Microbiology Letters **189**:215-218

Pratten J, Bedi R, Wilson M (2000b). An in vitro study of the effect of fluoridated milk on oral bacterial biofilms. Applied and Environmental Microbiology **66**:1720-1723

Pratten J, Wilson M, Spratt DA (2003). Characterisation of in vitro oral bacterial biofilms by traditional and molecular methods. Oral Microbiology and Immunology **18**:45-49

Quirynen M, Teughels W, De Soete M (2002). Topical antiseptics and antibiotics in the initial therapy of chronic adult periodontitis: microbiological aspects. Periodontology 2000 **28**:72-90

Raab O (1900). Uber die Wirkung fluoreszierender Stoffe auf Infusorien. Z. Biol. **39**:524-546

Ramberg P, Sekino S, Uzel NG, Socransky S, Lindhe J (2003). Bacterial colonization during de novo plaque formation. Journal of Clinical Periodontology **30**:990-995

Randazzo CL, Torriani S, Akkermans ADL, de Vos WM, Vaughan EE (2002). Diversity, dynamics and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16s rRNA analysis. Applied and Environmental Microbiology **68**:1882-1892

Rapley JW, Cobb CM, Killoy WJ, Williams DR (1992). Serum levels of tetracycline during treatment with tetracycline-containing fibers. Journal of Periodontology **63**:817-820

Reinholdt J, Kilian M (1987). Interference of IgA protease with the effect of secretory IgA on adherence of oral streptococci to saliva-coated hydroxylapetite. Journal of Dental Research **66**:492-497

Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS (2003). Bacterial coaggregation: an integral process in the development of multi-species biofilms. TRENDS in Microbiology **11**:94-100

Riep B, Purucker P, Bernimoulin JP (1999). Repeated local metronidazole – therapy as an adjunct to scaling and root planing in maintainance patients. Journal of Clinical Periodontology **26**:710-715

Roberts FA, Richardson GJ, Michalek SM (1997). Effects of *Porphyromonas gingivalis* and *Escherichia coli* lipopolysaccharide on mononuclear phagocytes. Infection and Immunity **65**:3248-3254

Roberts AP, Pratten J, Wilson M, Mullany P (1999). Transfer of a conjugative transposon Tn 5397 in a model oral biofilm. FEMS Microbiology Letters **177**:63-66

Rosan B, Lamont RJ (2000). Dental plaque formation. Microbes and Infection. **2**:1599-1607

Rossi E, Van De Vorst A, Jori G (1981). Competition between singlet oxygen and electron transfer mechanisms in the porphyrin-sensitized photooxidation of l-tryptophan and tryptamine in aqueous micellar dispersions. Photochemistry and Photobiology **34**:447-454.

Rotstein OD, Pruett TL, Fiegel VD, Nelson RD, Simmons RL (1985). Succinic acid, a metabolic by-product of *Bacteroides* species, inhibits polymorphonuclear leukocyte function. Infection and Immunity **48**:402-408

Rovaldi CR, Pievsky A, Sole NA, Friden PM, Rothstein DM, Spacciapoli P (2000). Photoactive porphyrin derivative with broad-spectrum activity against oral pathogens *in vitro*. Antimicrobial Agents and Chemotherapy **44**:3364-3367

Sakamoto M, Umeda M, Iskikawa I, Benno Y (2000). Comparison of the oral bacterial flora in saliva from a healthy subject and two periodontitis patients by sequence analysis of 16S rDNA libraries. Microbiology Immunology **44**:643-652

Salvi GE, Lawrence HP, Offenbacher S, Beck JD (1997). Influence of risk factors on the pathogenesis of periodontitis. Periodontology 2000 **14**:173-201

Santegoeds CM, Ferdelman TG, Muyzer G, De Beer D (1998). Structural and functional dynamics of sulphate-reducing populations in bacterial biofilms. Applied and Environmental Microbiology **64**:3731-3739

Saxton CA (1973). Scanning electron microscope study of the formation of dental plaque. Caries Research **7**:102-119

Scannapieco FA (1998). Position paper of the American academy of periodontology: Periodontal diseases as a potential risk factor for systemic diseases. Journal of Periodontology **69**:841-850

Schenkein HA, Genco RJ (1977). Gingival fluid and serum in periodontal diseases. Journal of Periodontology **48**:772-777

Schneider RP, Marshall KC (1994). Retention of the Gram-negative marine bacterium SW8 on surfaces – effects of microbial physiology, substratum nature and conditioning films. Colloids Surfaces B Biointerfaces **2**:387-396

Schreier H, Erdos G, Reimer K, Konig B, Konig W, Fleischer W (1997). Molecular effects of povidone-iodine on relevant microorganisms: an electron-microscopic and biochemical study. **195**(Suppl.2):111-116

Schuitmaker JJ, Baas P, Van Leengoed HLLM, Van der MeulenFW, Star WM, Van Zandwijk N (1996). Photodynamic therapy: a promising new modality for the treatment of cancer. Journal of Photochemistry and Photobiology B: Biology **34**:3-12

Schwank S, Rajacic Z, Zimmerli W, Blaser J (1998). Impact of biofilm formation on in vitro and *in vivo* activities of antibiotics. Antimicrobial Agents and Chemotherapy **42**: 895-898

Seal GJ, Ng Y-L, Spratt D, Bhatti M, Gulabivala K (2002). An in vitro comparison of the bactericidal efficacy of lethal photosensitization or sodium hypochlorite irrigation on Streptococcus intermedius biofilms in root canals. International Endodontic Journal **35**:268-274

Sismey-Durrant HJ, Hopps RM (1991). Effect of lipopolysaccharide from *Porphyromonas gingivalis* on prostaglandin E2 and interleukin -1β release from rat periosteal and human gingival fibroblasts in vitro. Oral Microbiology and Immunology **6**:378-380

Sissons CH, Wang L, Cutress TW (1995). Patterns and rates of growth of microcosm plaque biofilms. Oral Microbiology and Immunology. **10**:160-167

Skopek RJ, Liljemark WF, Bloomquist CG, Rodney JD (1993). Dental plaque development on defined streptococcal surfaces. Oral Microbiology and Immunology **8**:16-23

Slots J (1986). Rapid identification of important periodontal microorganisms by cultivation. Oral Microbiology and Immunology **1**:48-55

Smalla K, Wachtendorf U, Heuer H, Liu WT, Forney L (1998). Analysis of BIOLOG GN substrate utilisation patterns by microbial communities. Applied and Environmental Microbiology **64**:1220-1225

Socransky SS, Gibbons RJ, Dale AC, Bortnick L, Rosenthal E, MacDonald JB (1963). The microbiota of the gingival crevice area of man - I. Total microscopic and viable counts and counts of specific organisms. Archives of Oral Biology **8**:275-280.

Socransky SS (1970.) Relationship of bacteria to the aetiology of periodontal disease. Journal of Dental Research, **49** (Suppl.2):203-222

Socransky SS, Haffajee AD (1992). The bacterial etiology of destructive periodontal disease: current concepts. Journal of Periodontology **63**:322-331

Socransky SS, Haffajee AD (1993). Effect of therapy on periodontal infections. Journal of Periodontology **64**:754-759

Socransky SS, Haffajee AD (1994) Evidence of bacterial aetiology: Historical perspective. Periodontology 2000 **2**:1-7

Soukos NS, Wilson M, Burns T, Speight PM (1996). Photodynamic effects of toluidine blue on human oral keratinocytes and fibroblasts and *Streptococcus sanguis* evaluation in vitro. Lasers in Surgery and Medicine **18**:253-259

Soukos SS, Ximenez-Fyvie LA, Hamblin MR, Socransky SS, Hasan T (1998). Targeted antimicrobial photochemotherapy. Antimicrobial Agents and Chemotherapy **42**:2595-2601

Spikes JD (1985). The historical development of ideas on applications of photosensitised reaction in health sciences. In: Primary Photoprocesses in Biology and Medicine. Edited by Bergasson RV, Jori G, Land EJ, Truscott TG, Plenum Press, New York, pp209-227

Spratt DA, Weightman AJ, Wade WG (1999). Diversity of oral asaccharolytic *Eubacterium* species in periodontitis – identification of novel phylotypes representing uncultivated taxa. Oral Microbiology and Immunology **14**:56-59

Stoltze K, Stellfeld M (1992). Systemic absorption of metronidazole after application of a metronidazole 25 % dental gel. Journal of Clinical Periodontology **19**:693-697

Stoltze K (1995). Elimination of elyzol 25 % dental gel matrix from periodontal pockets. Journal of Clinical Periodontology **22**:185-188

Syed SA, Loesche WJ (1972). Survival of human dental plaque flora in various transport media. Applied Microbiology **24**:638-644

Takeuchi H, Yamamoto K (2001). Ultrastructural analysis of structural framework in dental plaque developing on synthetic carbonate apatite applied to human tooth surfaces. European Journal of Oral Sciences **109**:249-259

Tanner A, Maiden MFJ, Paster BJ, Dewhirst FE (1994). The impact of 16s ribosomal RNA-based phylogeny on the taxonomy of oral bacteria. Periodontology 2000 **5**:26-51

Tanner A, Maiden MFJ, Macuch PJ, Murray LL, Kent RL (1998). Microbiota of health, gingivitis and initial periodontitis. Journal of Clinical Periodontology **25**:85-98

Ter Steeg PF, Van Der Hoeven JS, De Jong MH, Van Munster PJJ, Jansen MJH (1988). Modelling the gingival pocket by enrichment of subgingival microflora in human serum chemostats. Microbial Ecology in Health and Disease **1**:73-84.

Theilade E, Fejerskov O, Karring T, Theilade J (1982). Predominant cultivable microflora of human dental fissure plaque. Infection and Immunity **36**:977-982

Theilade E (1986). The non-specific theory in microbial etiology of inflammatory periodontal diseases. Journal of Clinical Periodontology **13**:905-911

Tonetti M, Cugini M, Goodson J (1990). Zero-order delivery with periodontal placements of tetracycline loaded ethylene vinyl acetate fibers. Journal of Periodontal results **25**:243-249

Tonetti MS (1998). Local delivery of tetracycline: from concept to clinical application. Journal of Clinical Periodontology **25**:969-977

Travis J, Potempa J, Maeda H (1995). Are bacterial proteinases pathogenic factors? Trends in Microbiology **3**:405-407

Usacheva MN, Teichert MC, Biel MA (2001). Comparison of the Methylene Blue and Toluidine Blue photobactericidal efficacy against Gram-positive and Gram-negative microorganisms. Lasers in Surgery and Medicine **29**:165-173.

Valduga G, Bertolini G, Reddi E, Jori G (1993). Effect of extracellularly generated singlet oxygen on Gram-positive and Gram-negative bacteria. Journal of Photochemisitry and Photobiology B: Biology **21**:81-86.

Vandekerckhove BN, Quirynen M, Van Steenberghe D (1997). The use of tetracycline-containing controlled-release fibers in the treatment of refractory periodontitis. Journal of Periodontology **68**:353-361

Van Hillegersberg R, Kort WJ, Wilson P (1994). Current status of photodynamic therapy in oncology. Drugs **48**: 510-541

Van Oss CJ (1995). Hydrophobic, hydrophilic and other interactions in epitope-paratope binding. Molecular Immunology **32**:199-211

Van Palenstein Helderman WH (1981). Microbial aetiology of periodontal disease. Journal of Clinical Periodontology **8**:261-280

Van Winkelhoff AJ, Rams TE, Slots J (1996). Systemic antibiotic therapy in periodontics. Periodontology 2000 **10**:45-78

Verschuere L, Fievez V, Van Vooren L, Verstraete W (1997). The contribution of individual populations to the Biolog pattern of model microbial communities. FEMS Microbiology Ecology **44**:353-362

Von Tappeiner H (1900). Uber die Wirkung fluoreszierender Stoffe auf Infusorien nach Versuchen von O. Raab. Muench Med. Wochenschr **47**:5

Von Tappeiner H, Jodlbauer A (1904). Uber Wirkung der photodynamischen (fluorieszierenden) Stoffe auf Protozoan und Enzyme. Dtsch. Arch. Klin. Med. **80**:427-487

Von Tappeiner H, Jodlbauer A (1907). Die Sensibilisieren de Wirkung Fluorieszierender Substanzer. Gesammte Untersuchungen uber die photodynamische Erscheinung. F.C.W. Vogel, Leipzig.

Wagman GH, Bailey JV, Weinstein MJ (1975). Binding of aminoglycoside antibiotics to filtration materials. Antimicrobial Agents and Chemotherapy **7**:316-319

Wainwright M, Phoenix DA, Marland J, Wareing DRA, Bolton FJ (1997). A study of photobactericidal activity in the phenothiazinium series. FEMS Immunology and Medical Microbiology **19**:75-80.

Wainwright M, Phoneix DA, Laycock SL, Wareing DRA, Wright PA (1998a). Photobactericidal activity of phenothiazinium dyes against methicillin-resistant strains of *Staphylolcoccus aureus*. FEMS Microbiology Letters **160**:177-181.

Wainwright M (1998b). Photodynamic antimicrobial chemotherapy (PACT). Journal of Antimicrobial Chemotherapy. **42**:13-28

Wainwright M, Phoneix DA, Gaskell M, Marshall B (1999). Photobactericidal activity of methylene blue derivatives against vancomycin-resistant *Enterococcus* spp. Journal of Antimicrobial Chemotherapy **44**:823-825.

Wakayama Y, Takagi M, Yano K (1980). Photosensitised inactivation of E. coli cells in toluidine blue-light system. Photochemistry and Photobiology **32**: 601-605

Walker C. Antimicrobial agents and chemotherapy (1992). In: Slots J, Taubman MA, ed. Contemporary oral microbiology and Immunology. St. Louis: Mosby, pp242-346.

Walker CB (1996). Selected antimicrobial agents: mechanisms of action, side effects and drug interactions. Periodontology 2000 **10**:12-28

Watson TF (1991). Applications of confocal scanning optival microscopy to denistry. British Dental Journal. **171**(9):287-291

Wecke J, Kersten T, Madela K, Moter A, Gobel UB, Friedmann A, Bernimoulin JP (2000). A novel technique for monitoring the development of bacterial biofilms in human periodontal pockets. FEMS Microbiology letters **191**:95-101

Weiger R, Netuschil L, von Ohle C, Schlagenhauf U, Brecx M (1995). Microbial generation time during the early phases of supragingival dental plaque formation. Oral Microbiology and Immunology **10**:93-97

Weiger R, Von Ohle C, Decker E, Axmann-Kremar D, Netuschil L (1997). Vital microorganisms in early supragingival dental plaque and in stimulated human saliva. Journal of Periodontal Research **32**:233-240

Wiebe CB, Putnins EE (2000). The periodontal disease classification system of the American academy of periodontology - an update. J Can Dent Assoc **66**:594-597

Wilson M, Dobson J, Harvey W (1993a). Sensitisation of *Streptococcus sanguis* to killing by light from a helium/neon laser. Lasers in Medical Science. **8**:69-73

Wilson M, Dobson J, Sarkar S (1993b). Sensitisation of periodontopathogenic bacteria to killing by light from a low-power laser. Oral Microbiology and Immunology. **8**:182-187

Wilson M, Sarkar S, Bulman JS (1993c). Effect of blood on photosensitisation of bacteria in subgingival plaque from patients with chronic periodontitis. Lasers in Medical Science. **8**:297-303

Wilson M (1993d). Photolysis of oral bacteria and its potential use in the treatment of caries and periodontal disease. Journal of Applied Bacteriology **75**:299-306

Wilson M (1994a). Bactericidal effect of laser light and its potential use in plaque related diseases. International Dental Journal. **44**:181-189

Wilson M, Pratten J (1994b). Lethal photosensitisation of Staphylococcus aureus. Microbios. **78**:163-168

Wilson M, Pratten J (1994c). Sensitization *of Staphylococcus aureus* to killing by low-power laser light. Journal of Antimicrobial Chemotherapy **33**:619-624

Wilson M, Pratten J (1995a). Lethal photosensitisation of *Staphylococcus aureus* in vitro: Effect of growth phase, serum, and pre-irradiation time. Lasers in Surgery and Medicine **16**:272-276.

Wilson M, Kpendema J, Noar J, Hunt N, Mordan N (1995b). Corrosion of intra-oral magnets in the presence and absence of *Streptococcus sanguinis* biofilms. Biomaterials **16**:721-725

Wilson M, Burns T, Pratten J, Pearson GJ (1995c). Bacteria in supragingival plaque samples can be killed by low-power light in the presence of a photosensitizer. Journal of Applied Bacteriology **78**:569-574

Wilson M, Burns T, Pratten J (1996a). Sensitisation of *Streptococcus sanguis* biofilms using a light-activated antimicrobial agent. Journal of Antimicrobial Chemotherapy. **37**:377-381

Wilson M (1996b) Susceptibility of oral bacterial biofilms to antimicrobial agents. Journal of Medical Microbiology **44**:79-87

Wilson M, Reddi K, Henderson B (1996c). Cytokine-inducing components of periodontopathogenic bacteria. Journal of Periodontal Results **31**:393-407

Wilson M, Patel H, Noar JH (1998). Effect of chlorhexidine on multi-species biofilms. Current Microbiology **36**:13-18

Wilson M (2001). Bacterial biofilms and human disease. Science Progress **84**:235-254

Wimpenny J (1988) Introduction In:Wimpenny J (Ed) Handbook of laboratory model systems for microbial ecosystems. CRC Press, Boca Raton, Fl, pp1-17

Wimpenny J, Manz W, Szewzyk U (2000). Heterogeneity in biofilms. FEMS Microbiology Reviews. **24**:661-671

Wood S, Nattress B, Kirkham J, Shore R, Brookes S, Griffiths J, Robinson C (1999). An in vitro study of the use of photodynamic therapy for the treatment of natural oral plaque biofilms formed in vivo. Journal of Photochemistry and Photobiology B:Biology **50**:1-7

Wood SR, Kirkham J, Marsh PD, Shore RC, Nattress B, Robinson C (2000). Architecture of intact natural human plaque biofilms studied by confocal laser scanning microscopy. Journal of Dental Research **79**:21-27

Xie H, Cook GS, Costerton JW, Bruce G, Rose TM, Lamont RJ (2000). Intergeneric communication in dental plaque biofilms. Journal of Bacteriology **182**(24):7067-7069

Ximenez-Fyvie LA, Haffajee AD, Socransky SS (2000). Microbial composition of supra- and subgingival plaque in subjects with adult periodontitis. Journal of Clinical Periodontology **27**:722-732

Yamaji Y, Kubota T, Sasaguri K, Sato S, Suzuki Y, Kumada H, Umemoto T (1995). Inflammatory cytokine gene expression in human periodontal ligament fibroblasts stimulated with bacterial lipopolysaccharides. Infection and Immunity **63**:3576-3581

Zee KY, Samaranayake LP, Attstrom R (1996). Predominant cultivable supra-gingival plaque in chinese "rapid" and "slow" plaque formers. Journal of Clinical Periodontology **23**:1025-1031

Zeina B, Greenman J, Purcell WM, Das B (2001). Killing of cutaneous microbial species by photodynamic therapy. British Journal of Dermatology. **144**:244-278

Zhang XQ, Bishop PL, Kupferle MJ (1998). Measurement of polysaccharides and proteins in biofilm extracellular polymers. Water sci technol. **37**:345-348

Zijinge V, Harmsen HJM, Kleinfelder JW, Van Der Rest ME, Degener JE, Welling GW (2003). Denaturing gradient gel electrophoresis analysis to study bacterial community structure in pockets of periodontal patients. Oral Microbiology and Immunology **18**:59-65

Zinner DD, Aran AP, Saslaw MS, Jablon JM, Fitzgerald RJ (1967). Induction of dental caries in gnotobiotic rats by Streptococci of human origin. Nature **213**:200-1

Zylber LJ, Jordan HV (1982). Development of a selective medium for detection and enumeration of Actinomyces viscosus and Actinomyces naeslundii in dental plaque. Journal of Clinical Microbiology **15**:253-259

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Oral Bacteria in Multi-Species Biofilms Can be Killed by Red Light in the Presence of Toluidine Blue

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Background and Objectives: Oral bacteria can be killed by light in the presence of a suitable photosensitizer, and this could be used in the treatment of oral infections. In these diseases, however, bacteria are present as biofilms, which are refractive to antimicrobial agents. The purpose of this study was to determine whether oral bacterial biofilms were susceptible to lethal photosensitization.
Study Design/Materials and Methods: Multi-species biofilms of oral bacteria were irradiated with light from a helium/neon laser in the presence of toluidine blue O (TBO) and the survivors enumerated. Controls examining the effects of light and TBO alone were also included. The biofilms were also examined by confocal scanning laser microscopy (CSLM).
Results: CSLM revealed that the biofilms had structures similar to those of dental plaque. Although, the biofilms consisted of extremely large numbers of bacteria ($\sim 9 \times 10^9$), 97.4% were killed following irradiation with 31.5 J of laser light in the presence of 25 μg/ml TBO.
Conclusions: Substantial numbers of oral bacteria in multi-species biofilms can be killed by light in the presence of TBO. This may be useful in the treatment of dental plaque-related diseases. Lasers Surg. Med. 31:86–90, 2002. © 2002 Wiley-Liss, Inc.

Key words: lethal photosensitization; biofilms; dental plaque; caries; periodontitis; toluidine blue

INTRODUCTION

The use of light-activated antimicrobial agents represents an alternative approach to killing bacteria, which in view of the growing resistance of many organisms to conventional antimicrobials, may prove to be of great importance in the future [1]. Such agents (e.g., hematoporphyrins, phthalocyanines, toluidine blue O (TBO)) are activated by irradiation with light of an appropriate wavelength resulting in the generation of cytotoxic species, including singlet oxygen and free radicals, which can exert a bactericidal effect [2]. Agents of this type have been used for several years to destroy various kinds of tumor in vivo and interest has been growing in applying this approach to treating infectious diseases. For example, hematoporphyrins in conjunction with light from an Argon laser have been used to treat post-neurosurgical infections [3] and brain abscesses [4] in man. Oral infections would appear to be particularly amenable to treatment with light-activated agents as they are essentially topical infections, which are readily accessible by both the agent and light. However, the target organisms in these infections are present within biofilms (dental plaques), which are notoriously resistant to a wide range of antimicrobial agents [5,6]. A biofilm consists of an accumulation of bacteria and their extracellular products on a surface and the plaques that form on tooth surfaces are typical examples of such structures [6]. While the susceptibility to lethal photosensitization of mono-species biofilms has been reported [7], there is little information on the susceptibility of biofilms consisting of a range of different bacteria. The purpose of this study was to determine the susceptibility to lethal photosensitization of multi-species biofilms with a bacterial composition similar to those, which exist in the human oral cavity.

MATERIALS AND METHODS

Preparation of Multi-Species Biofilms

Saliva samples (3 ml) were obtained from 10 healthy adult volunteers and pooled. Thirty milliliters of Brain heart infusion broth (Oxoid Ltd., Basingstoke, United Kingdom) containing 10% (w/v) glycerol (Sigma Ltd., Poole, United Kingdom) were added as a cryoprotectant and the mixture was vortex mixed for 1 minute. One-milliliter aliquots were then dispensed into sterile cryotubes, which were then stored in a freezer at – 70°C.

Multi-species biofilms were grown on 5-mm diameter discs prepared from cellulose nitrate membrane filters with a pore diameter of 0.45 μm (Sterilin Ltd., Hounslow, United Kingdom). The biofilms were produced as follows. A cryotube containing pooled saliva was removed from the – 70°C freezer and allowed to defrost. Sterile 5-mm diameter cellulose nitrate discs were then placed on the surface of fastidious anaerobe agar (FAA; Lab M, Bury, United Kingdom). Each disc was inoculated with 25 μl of

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the saliva suspension. The plates were then placed in an anaerobic cabinet (Don Whitley Scientific Ltd., Shipley, United Kingdom) for 24 hours.

Susceptibility of Biofilms to Lethal Photosensitization

FAA plates containing discs with associated biofilms were removed from the anaerobic cabinet 24 hours after seeding of the cellulose nitrate membranes. Ten microliters of 25 μg/ml TBO (Zila, Inc., Phoenix, Arizona) in saline (0.85% w/v) was added to one of the biofilms (designated L+S+). After 30 seconds, the biofilm was exposed for 15 minutes to light (632 nm) from a Helium/neon (He/Ne) gas laser with a measured power output of 35 mW (Spectra-physics, Darmstadt-Kranichstein, Germany). The total energy dose was 31.5 J and the energy density 81.9 Jcm^{-2}. The disc was then placed in 1 ml of sterile nutrient broth and vortexed for 60 seconds. The number of viable organisms surviving within the biofilm was then determined by viable counting as described below. The same procedure was then repeated for a second biofilm.

In order to determine the effect of laser light alone on bacterial viability, 10 μl of saline (0.85% w/v) was added to another two biofilms (designated L+S−) and, after 30 seconds, each of the biofilms was exposed to laser light as described above. Other controls consisted of biofilms treated with TBO, but not exposed to laser light (L−S+) and biofilms, which were neither treated with TBO nor exposed to laser light (L−S−).

The whole procedure described above was repeated on three separate occasions.

Bacteriological Analysis

A non-selective medium was used to determine the total number of viable bacteria in the biofilms. Selective media were used to determine the number of viable bacteria belonging to the major groups of bacteria initially present in the biofilms. Serial dilutions of the disrupted biofilms in nutrient broth were prepared in nutrient broth and duplicate 50 μl aliquots from each dilution were spread over the surfaces of the following media: (i) FAA plates—for enumerating organisms able to grow anaerobically, (ii) FAA plus 2.5 μg/ml vancomycin and 10 μg/ml nalidixic acid (FAX)—for Gram-negative anaerobes, (iii) Mitis salivarius (MS) agar (Difco, Detroit, Michigan)—for streptococci, (iv) Veillonella agar (Difco)—for *Veillonella* species, and (v) Cadmium fluoride-acriflavin-tellurite (CFAT) agar—for *Actinomyces* species.

All plates were incubated in an anaerobic cabinet at 37°C for 7 days.

The resulting colonies growing on the non-selective medium were counted. Representative colonies on the selective media were Gram stained and those displaying the following characteristics were counted: (i) Gram-negative cocci or bacilli on the FAX medium—these were considered to be Gram-negative anaerobes, (ii) Gram-positive cocci on the MS medium—these were considered to be streptococci, (iii) Gram-negative cocci on the veillonella agar—these were considered to be *Veillonella* spp., and (iv) Gram-positive rods on the CFAT agar—these were considered to be *Actinomyces* spp.

Confocal Laser Scanning Microscopy (CLSM)

The membrane filters supporting the biofilms were placed into a miniature petri dish, biofilm upward. Ten milliliters of chilled phosphate-buffered saline containing 10 μl each of component A and B (*Bac*Light™ bacterial viability kit, Molecular Probes, Eugene, Oregon) was carefully added to the petri dish and allowed to cover the discs. This was allowed to stand in the dark to allow the stain to develop for 10 minutes. Scans were taken of the biofilms using a Leica DMLFS fixed stage microscope with a Leica TCS SP confocal laser scan-head. The objective lens was an infinity corrected 63× HCX water immersion dipping objective lens (Leica, Milton Keynes, Northamptonshire, United Kingdom). The *z* step size used in these experiments was 1 μm.

The CLSM image stack data were analyzed by Leica Confocal Software Simulator SP (version 2.00) (Leica Microsystems Heidelberg GmbH, Germany) to produce surface renderings. The image stack (biofilm) topography was quantified before a three-dimensional rendering of the surface could be constructed. The surface topography data were processed in order to allow macroscopic features of the biofilm architecture to be seen in the surface renderings without being masked by the finer surface details of the image stack. The topographical data underwent a 'leveling' process (linear interpolation function to reduce waviness) followed by the application of a 'threshold' to the rendering process (data values that are below the specified value are ignored by the calculation of the topographical image). The final surface renderings were compared to the projection images to ensure they accurately represented the macroscopic biofilm architecture.

RESULTS

The biofilms consisted of 32.8% streptococci, 6.5% *Veillonella* spp., 0.1% *Actinomyces* spp., and 0.1% Gram-negative anaerobes. The biofilms contained very large numbers of viable bacteria, the total viable count being $8.73 \times 10^9 \times 4.88 \times 10^9$ colony-forming units (cfu), which equates to a bacterial density of 4.45×10^{10} cfu/cm^2. A representative CLSM image of a biofilm prior to lethal photosensitization is shown in Figure 1. Figure 1a shows the distribution of live bacteria (as revealed by the live/dead staining procedure), while Figure 1b shows the distribution of the dead bacteria. The flat plane in these images is not the surface of the membrane filter, it is a plane constructed by the computer as a result of the leveling and threshold filters applied to the CLSM data as described in the Materials and Methods. A large number of tower-like "stacks" (structures typical of biofilms) can be seen in the live channel and the three most substantial of these have been labeled. The corresponding image showing the dead bacteria (Fig. 1b) shows that only stack no. 3 gives an appreciable signal, i.e., only this stack contains appreciable numbers of dead bacteria. Image analysis

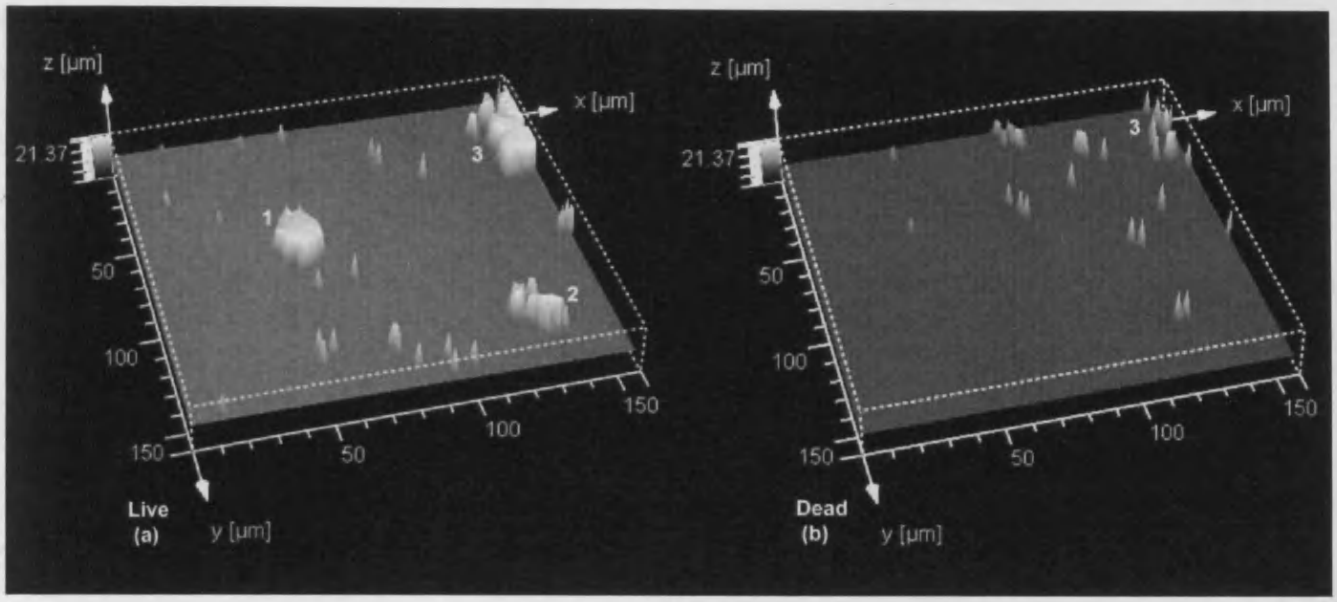

Fig. 1. Confocal laser scanning micrograph of a biofilm prior to exposure to either laser light or TBO. The biofilm was stained using the *Bac*Light live/dead procedure. (**a**) The distribution of live bacteria in the biofilm. (**b**) The distribution of dead bacteria. Prominent "stacks" in the biofilm are numbered.

measurements of the pixel density of the green (i.e., live) and red (i.e., dead) images revealed that the relative proportion of live and dead bacteria was 2.6:1.

Lethal photosensitization of the biofilms with TBO at a concentration of 25 µg/ml and a light energy dose of 31.5 J resulted in the killing of 97.4% of the bacteria, i.e., $\sim 8.5 \times 10^9$ cfu. This reduction was statistically significant (two-tail t-test assuming unequal variance, $P = 0.0002$). A total of 26.9% of the bacteria in the biofilms were killed by exposure to laser light in the absence of TBO, but none were killed by TBO in the absence of laser light. However, the reduction in viable counts achieved by the laser light in the absence of TBO was not statistically significant ($P = 0.327$). CLSM images of a representative biofilm following exposure to laser light in the presence of TBO are presented in Figure 2. Three stacks (no. 1–3) are visible in the dead channel (Fig. 2b) that are not visible in the live channel (Fig. 2a). A larger stack (no. 4) is visible in both the live and dead channels. In this stack, the dead volume is larger than that of the live volume suggesting that the outer layer is dead with only the interior producing a live signal. Measurements of the pixel density of the green (i.e., live) and red (i.e., dead) images revealed that the relative proportion of live and dead bacteria was 0.3:1.

DISCUSSION

A number of studies have shown that oral bacteria are very susceptible to lethal photosensitization when they are suspended in an aqueous medium [1,8,9]. However, the causative agents of the most prevalent infectious diseases of the oral cavity—caries and periodontal diseases—are present in biofilms on the surface of teeth. It has been known for many years that biofilm-grown cells differ from their planktonic counterparts in a number of respects including: (a) cell wall composition, (b) rate of growth, (c) metabolic activity, and (d) gene expression [10,11]. Furthermore, bacteria within a biofilm are enclosed within a matrix of polymeric material, which may serve to protect them against adverse environmental factors, including antimicrobial agents [6,11,12]. As a result of the above-mentioned differences, bacterial biofilms display considerably reduced susceptibility to antimicrobial agents compared to their planktonic counterparts [11,13,14]. When considering the use of light-activated agents for the treatment of dental plaque-related diseases, it is important, therefore, that account be taken of the fact that the bacteria responsible are present as biofilms and so are likely to have reduced susceptibility to such agents. Few studies appear to have addressed this issue. It has been shown previously that biofilms of the important plaque-forming organism, *Streptococcus sanguis*, can be killed using light-activated aluminum disulphonated phthalocyanine [7]. However, the light energy dose necessary to kill substantial proportions of the organism in the biofilm was considerably higher (~15 fold) than that required to kill the organism when it is in an aqueous suspension. In a study involving multi-species biofilms similar to those that would be found in vivo, Wood et al. [15], reported that "widespread cell killing" occurred (although this was determined microscopically and the extent of killing was not quantified) when the biofilms were treated with pyridinium zinc (II) phthalocyanine and exposed for 30 minutes to a 400 W tungsten filament lamp. The

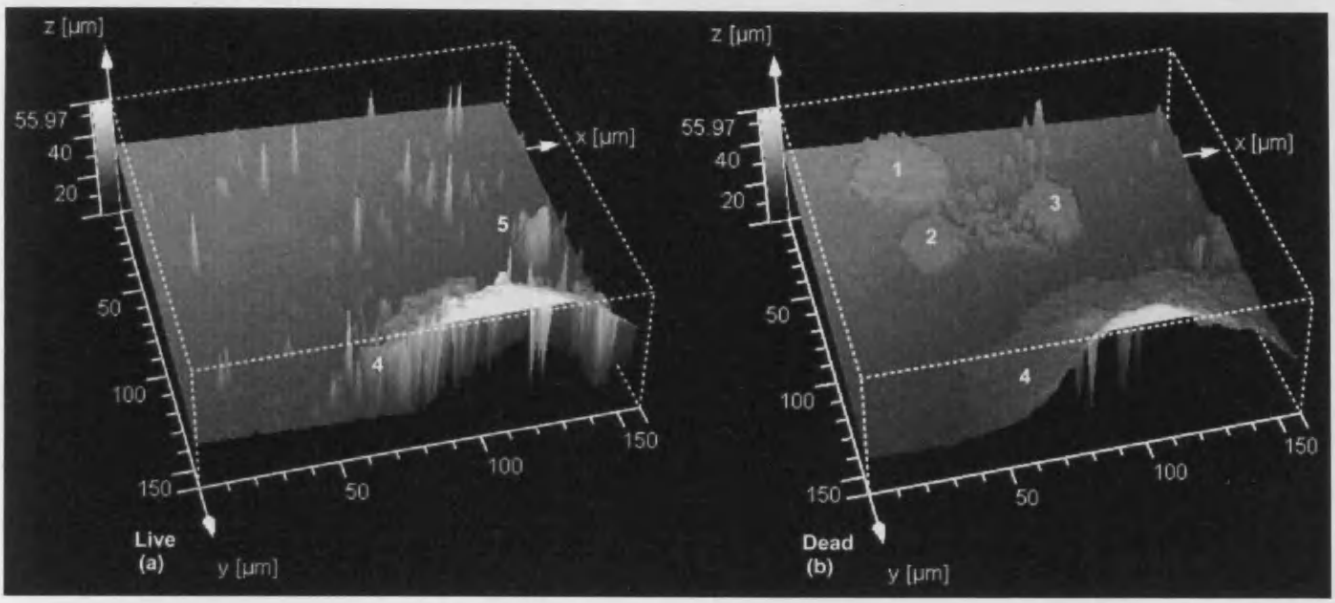

Fig. 2. Confocal laser scanning micrograph of a biofilm after irradiation with 31.5 J of light (632 nm) from a Helium/neon laser in the presence of TBO (25 µg/ml). The biofilm was stained using the *Bac*Light live/dead procedure. (**a**) The distribution of live bacteria in the biofilm. (**b**) The distribution of dead bacteria. Prominent "stacks" in the biofilm are numbered.

results of the present study have shown that very large numbers ($\sim 9 \times 10^9$ cfu) of bacteria present in multi-species oral biofilms derived from human saliva can be killed when treated with TBO and irradiated with red light (81.9 Jcm^{-2}). The CLSM analysis revealed that the biofilms used in the study had structures similar to those of biofilms found in vivo [16]. Furthermore, CLSM images of the biofilms after exposure to laser light in the presence of TBO revealed that in some of the biofilm stacks, lethal photosensitization occurred predominantly in the outer layers of the stack leaving some of the innermost bacteria alive. This could be due to the inability of the photosensitizer to diffuse through into these inner regions, the inability of the light to penetrate into these regions, or a combination of both. This illustrates one potential problem associated with the photodynamic therapy (PDT) of biofilm-related diseases. However, this could be overcome by selecting a photosensitizer able to penetrate through the biofilm matrix and by irradiating biofilms internally (e.g., via an optical fiber inserted into the biofilm itself) rather than from the biofilm surface.

The essentially topical nature of dental plaque-related infections renders them amenable to PDT. Systemic administration of the photosensitizing agent would not be necessary as it could be applied directly to the lesion and access of the light to the lesion would not require invasive measures—an optical fiber could be used to guide the light beam onto/into the lesion. PDT would circumvent some of the well-recognized disadvantages associated with the use of conventional antimicrobial agents for the treatment of oral infections. Hence, resistance development would be unlikely as killing is mediated by singlet oxygen and free radicals and killing is attained very rapidly (within minutes) obviating the need to maintain high concentrations of chemicals in the disease lesion for long periods of time. Furthermore, treatment can be confined to the site of the lesion by careful topical application of the photosensitizer and by controlling the orientation and diameter of the light beam thereby minimizing the chances of disrupting the resident microflora at other sites. One potential side effect of this antibacterial approach, which needs to be considered is the possibility of damage to host tissues. Studies in animals [17,18], however, have shown no evidence of damage to host tissues even when using light doses and TBO concentrations much higher than those required to achieve a bactericidal effect.

In conclusion, the results of this study have shown that substantial numbers of oral bacteria in multi-species biofilms can be killed by light in the presence of TBO suggesting that this approach may be useful in the treatment of dental plaque-related diseases.

REFERENCES

1. Wilson M. Photolysis of oral bacteria and its potential use in the treatment of caries and periodontal disease: A review. J Appl Bact 1993;75:299–306.
2. Malik Z, Hanania J, Nitzan Y. Bactericidal effects of photoactivated porphyrins—An alternative approach to antimicrobial drugs. J Photochem Photobiol 1990;5:281–293.
3. Lombard GF, Tealdi S, Lanotte MM. The treatment of neurosurgical infections by lasers and porphyrins. In: Jori G, Perria CA, editors. Photodynamic therapy of tumours and other diseases. Padova: Edizioni Libreria Progetto. 1985: 363–366.
4. Laws ER, Wharen RE, Anderson RE. Photodynamic therapy of brain abscesses. In: Jori G, Perria CA, editors. Photodynamic therapy of tumours and other diseases. Padova: Edizioni Libreria Progetto. 1985:311–316.

5. Nichols WW. Sensitivity of bacteria in biofilms to antibacterial agents. In: Denyer SP, Gorman SP, Sussman M, editors. Microbial biofilms: Formation and control. Oxford: Blackwell Scientific Publications Ltd. 1993:187–200.
6. Wilson M. Susceptibility of oral bacterial biofilms to antimicrobial agents. J Med Microbiol 1996;44:79–87.
7. Wilson M, Burns T, Pratten J. Killing of *Streptococcus sanguis* in biofilms using a light-activated antimicrobial agent. J Antimicrob Chemother 1996;37:377–381.
8. Rovaldi CR, Pievsky A, Sole NA, Friden PM, Rothstein DM, Spacciapoli P. Photoactive porphyrin derivative with broad-spectrum activity against oral pathogens in vitro. Antimicrob Agents Chemother 2000;44:3364–3367.
9. Soukos NS, Ximenez-Fyvie LA, Hamblin MR, Socransky SS, Hasan T. Targeted antimicrobial photochemotherapy. Antimicrob Agents Chemother 1998;42:2595–2601.
10. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: A common cause of persistent infections. Science 1999;284: 1318–1322.
11. Wilson M. Bacterial biofilms and human disease. Science Progress 2001;84:235–254.
12. Anwar H, Strap JL, Costerton JW. Establishment of aging biofilms: Possible mechanism of bacterial resistance to antimicrobial chemotherapy. Antimicrob Agents Chemother 1992;36:1347–1351.
13. Lewis K. Riddle of biofilm resistance. Antimicrob Agents Chemother 2001;45:999–1007.
14. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 2001;9:34–39.
15. Wood S, Nattress B, Kirkham J, Shore R, Brookes S, Griffiths J, Robinson C. An in vitro study of the use of photodynamic therapy for the treatment of natural oral plaque biofilms formed in vivo. J Photochem Photobiol B 1999;50:1–7.
16. Netuschil L, Reich G, Unteregger G, Sculean A, Brecx M. A pilot study of confocal laser scanning microscopy for the assessment of undisturbed dental plaque vitality and topography. Arch Oral Biol 1998;43:277–285.
17. Millson CE, Thurrell W, Buonaccorsi G, Wilson M, MacRobert AJ, Bown SG. The effect of low-power laser light at different doses on gastric mucosa sensitised with methylene blue, haematoporphyrin derivative or toluidine blue. Lasers Med Sci 1997;12:145–150.
18. Komerik N, Speight P, Curnow A, Postle-Hacon M, Wilson M, Hopper C. The effect of photodynamic therapy on rat buccal mucosa. J Dent Res 1998;77:978.

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Comparative Antistreptococcal Activity of Photobactericidal Agents

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Summary

In order to establish a comparative order of efficacy among established photosensitising compounds currently under investigation, the *in vitro* photobactericidal activities of six commercially available photosensitisers were investigated at equal concentration against *Streptococcus sanguis* using a Helium Neon (HeNe) laser (632.8 nm).

Of the photosensitisers used, the four phenothiazinium compounds were efficient photobactericidal agents as was the protoporphyrin IX salt. However, the zinc phthalocyaninetetrasulfonate was less effective. Of the active agents, 1,9-dimethyl Methylene Blue (DMMB) was notable in achieving complete bacterial kill when used at a concentration of 40.85 μM in conjunction with a light energy dose of 21.8 J cm^{-2}, although there was inherent dark activity associated with this compound.

Since each of the photosensitisers is well known to produce singlet oxygen, the relative activities exhibited are thought to be due to differences in bacterial cell uptake, which in turn are related to the physicochemical properties of the photosensitisers, in particular, to the combination of lipophilicity and ionic character.

Key words: **Antibacterial, disinfection, photosensitisers, *Streptococcus sanguis*.**

INTRODUCTION

Today, drug-resistant strains of common pathogenic bacteria are a significant cause of mortality in our hospitals. The rise of the hospital "superbug" is a particular cause for grave concern in view of the paucity of effective conventional antimicrobial agents. Indeed, the rate of development of resistance to newly introduced agents is rapid [1]. Government committee reports, e.g. in the UK, have emphasised both the need for clean practices within the healthcare milieu as a barrier to infection and for more stringent conservation of effective antimicrobials [2], but the fact remains that hospitals are highly effective breeding grounds for drug-resistant pathogens.

The popularly perceived miraculous nature of antibiotics and modern antibacterial agents often lies in the fact that they are effective systemic drugs. However, the collateral effects on the commensal microflora have until recently been mainly disregarded and this attitude has aided the spread of bacterial resistance. The use of topical antisepsis is logically much less harmful to the body's indigenous microflora.

It is thus sensible to develop effective strategies for disinfection, both of wounds and of the carrier state, outside the field of conventional agents. A topical approach to disinfection would also allow greater conservation of effective systemic drugs for life-threatening conditions such as septicaemia.

Photodynamic antimicrobial chemotherapy (PACT [3]) is a potential approach to topical disinfection, employing photosensitising drugs and directed low-power light to produce reactive oxygen species (ROS) such as the hydroxyl radical, singlet oxygen

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etc. *in situ* [3]. Targeting of microbial species is possible by chemical design and also by the direction of illumination at the infected/colonised site. The production of ROS at the microbial target then leads to rapid oxidative damage and cell death via a range of mechanisms, depending on the nature of the target (e.g. bacterial cell wall/membrane, viral nucleic acid, enzymes etc.) [3]. In addition, the high reactivity of ROS such as singlet oxygen means that it is unlikely that there is a single target for any given photobactericidal agent, and this makes the development of resistance via target alteration more difficult. Singlet oxygen is also known to inactivate antioxidant enzymes such as catalase and superoxide dismutase [4]. Photobactericidal resistance due to elevated efflux has not yet been described.

The vital stains Methylene Blue (MB) and Toluidine Blue O (TBO), *inter alia*, have been used successfully by the authors as lead compounds in photoantimicrobial research over the past decade, both against standard and drug-resistant bacteria [5,6]. Other photosensitisers proposed for PACT have been developed in the parallel field of cancer photodynamic therapy (PDT) and are based on the porphyrin nucleus. While there is perhaps less rationale for their use in disinfection compared to the vital stains mentioned above, various groups have nevertheless reported activity in this area, in particular against Gram-positive bacteria [6]. However, considering the wide range of candidate photosensitisers available, little has been reported concerning the best type of photosensitiser for antimicrobial application.

The present work represents an investigation into the comparative activities of a range of established photosensitisers, both cationic and anionic and of different chemical type, against a standard bacterial species. *Streptococcus sanguis* is a Gram-positive organism which is commonly found in dental plaque [5], and can cause serious disease such as endocarditis [7].

MATERIALS AND METHODS

Photosensitisers

The photosensitisers, Methylene Blue (MB), New Methylene Blue (NMB), and dimethyl Methylene Blue (DMMB) were all purchased from Aldrich (Gillingham, UK) and were purified by recrystallisation from methanol. Toluidine Blue O (TBO), purchased from Zila Inc. (Arizona, USA), zinc phthalocyaninetetrasulfonic acid, tetrasodium salt (ZnTSPc) and protoporphyrin IX, disodium salt (PPIX) from Frontier Scientific (Lancaster, UK) were used without further purification.

Screening methodology

The organism used in this study was *Streptococcus sanguis* NCTC 7863. For experimental purposes the organism was grown in tryptone soya broth (Oxoid Ltd, Basingstoke U.K) at 37°C in an anaerobic cabinet overnight. The bacterial culture was diluted in saline (0.85%) to give an optical density of approximately 0.2 at 560 nm, 100 µl aliquots of this bacterial suspension were transferred to wells of a 96-well, round-bottomed micro-titre plate (Sterilin Ltd., Hounslow, UK). An equal volume of the filter-sterilised solution of the photosensitiser was added to some of the wells (4 for each photosensitiser) to give a final concentration of 40.85 µM. 4.0 mm magnetic stirrer bars were added to each well. The plate was placed on a magnetic stirrer, the wells mixed for 30 s and half of the wells (2 for each photosensitiser) then exposed to laser light for 60 seconds. The laser used was a Helium/Neon (He/Ne) gas laser with a measured power output of 35 mW (Spectra-Physics, Darmstadt-Kranichstein, Germany). This emits radiation in a collimated beam, diameter 3.5 mm, with a wavelength of 632.8 nm. Suspensions treated in this way were designated L+S+. The remaining wells (2 for each photosensitiser) were not exposed to laser light and these served as controls to determine the dark toxicity (L-S+). Control wells (2) containing the bacterial suspension plus sterile saline were also irradiated for 60 seconds to determine the effect of laser irradiation alone on bacterial viability (L+S-). Illuminated wells (both L+S+ and L+S-) thus received a light dose of 21.8 J cm^{-2} (= 0.035 W x 60 s / $\pi[0.35/2]^2$ cm^2). A further 2 wells, identical to those described above, were prepared and these were not exposed to laser light (L-S-).

The number of viable organisms surviving in each well was then determined by viable counting. Duplicate 50µl aliquots from the appropriate 10-fold dilutions were spread over the surface of Tryptone soya agar plates (Oxoid Ltd,). These plates were incubated in an anaerobic cabinet (Don Whitley) at 37°C. The resulting colonies were counted.

Singlet oxygen production

The photosensitisers (30 µM in methanol) were assayed for efficiency of singlet oxygen production using the decolourisation of the singlet oxygen quenching agent, 1,3-diphenylisobenzofuran (DPIBF, in methanol). Thus, to 3 ml of photosensitiser solution in a 1 cm quartz cuvette was added a sufficient volume of DPIBF solution (ca 0.2 ml) to give an absorbance of 1.8 units at 410 nm (Perkin Elmer Lambda 15 UV/Vis spectrophotometer). Cuvettes containing the different photosensitisers were placed in a carousel and illuminated for periods of 30 s using a slide projector lamp (Viewproject, NOBO,

UK) at a distance of 20 cm. The decrease in absorption at 410 nm was monitored spectrophotometrically with time. By assuming that the decrease in absorption of DPIBF at 410 nm is directly proportional to its reaction with singlet oxygen, the time for a 50% decrease in absorption caused by each of the photosensitisers under identical conditions ($t_{½PS}$) thus gives a measure of its ability to generate singlet oxygen which is considered to be the main bactericidal species produced by photosensitisers. Thus, the time for the DPIBF absorption to decrease by 50% due to MB photosensitisation ($t_{½MB}$) was taken as 1.0. The singlet oxygen yield for the photosensitisers ($\Phi_{\Delta PS}$), was calculated using the formula

$$\Phi_{\Delta PS} = \Phi_{\Delta MB} \bullet (t_{1/2MB} / t_{1/2PS})$$

Determinations were repeated three times.

Log P

The lipophilicities of the photosensitisers were calculated in terms of log *P*, the logarithm of their partition coefficients between phosphate-buffered saline and 1-octanol. The data were calculated using the standard spectrophotometric method [3], based on the relationship:

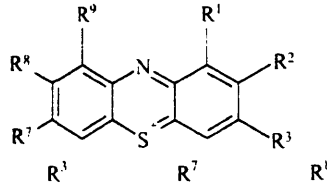
$$LogP = Log \frac{(A - A^1)}{A^1} \frac{V_w}{V_O}$$

where A and A^1 are the absorption intensities before and after partitioning respectively and V_w and V_o are the respective volumes of the aqueous and 1-octanol phases. Determinations were repeated three times.

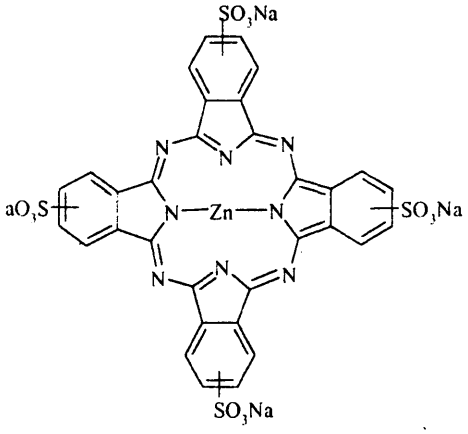
RESULTS

The photosensitisers used in the study are shown in *Figure 1*. Four of the compounds TBO, PPIX, NMB and ZnTSPc, exhibited maximum wavelengths of light absorption at around 630 nm, being compatible with the HeNe laser output (632.8 nm), although they exhibited a range of efficiencies for the production of singlet oxygen *in vitro* (*Table 1*). Although MB and DMMB usually exhibit maximum absorption peaks at somewhat longer wavelengths (ca. 650-660 nm), both absorb strongly in the region 620-640 nm (approx. 75-80% of the A_{max}). In addition, it was found that on mixing with the bacterial suspension the absorption at the lower wavelength range increased.

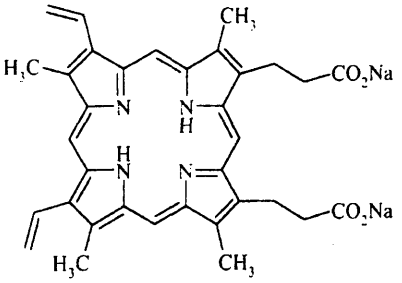
With the exception of zinc phthalocyaninetetrasulfonate, these compounds achieved appreciable kills (> 2 log reduction) of the organisms when irradiated with HeNe laser light (*Figure 2*). In addition, dimethyl Methylene Blue exhibited dark toxicity and also exhibited the greatest photobactericidal effect of the series used. In the latter example, the photokill



	R^1	R^2	R^3	R^7	R^8	R^9
MB	H	H	$N(CH_3)_2$	$N(CH_3)_2$	H	H
TBO	H	CH_3	$N(CH_3)_2$	NH_2	H	H
NMB	H	CH_3	$NHCH_2CH_3$	$NHCH_2CH_3$	CH_3	H
DMMB	CH_3	H	$N(CH_3)_2$	$N(CH_3)_2$	H	CH_3



ZnTSPc



PPIX

FIGURE 1 - Photosensitiser structures.

was complete – no viable organisms were detectable (*Figure 2*) – but high levels of photokilling have been reported previously with this photosensitiser using much lower concentrations [9]. The order of photobactericidal efficacy observed against *S. sanguis* was: MMB>NMB>TBO>MB>PPIX>>ZnTSPc

TABLE 1 - *Comparative singlet oxygen generating abilities and lipophilicities of the photosensitisers.*

	$^1O_2$ relative yield	[a]Log*P*
MB	1.00	-0.10
ZnTSPc	0.93	-[b]
DMMB	1.22	+1.01
NMB	1.35	+1.20
TBO	0.86	-0.21
PPIX	1.08	+3.19

[a]Log*P* is a measure of the partitioning behaviour of the photosensitisers between phosphate buffer and 1-octanol. [b]ZnTSPc was not detectable in the octanol layer.

Bacterial suspensions which were incubated without photosensitisers exhibited little or no decrease in viable counts with or without irradiation (*Figure 2*), thus demonstrating that the light source alone (632.8 nm, 21.8 J cm^{-2}) had no cytotoxic effect at the energy dose used in this study.

In terms of hydrophilic / lipophilic characteristics, the compounds followed an order of increasing lipophilicity (*Table 1*), ZnTSPc <<TBO <MB (hydrophilic Log *P* <0) <DMMB <NMB <PPIX (lipophilic, Log *P* >0).

DISCUSSION

Several groups have reported the photobactericidal effect of various photosensitisers but such work has been concerned with single or closely-related compounds rather than examining different structural types [10,11]. In the present investigation the series of compounds used furnished a range of chemical types (phenothiazinium / porphyrin / phthalocyanine), hydrophilicities and covered both anionic and cationic species.

The photodynamic efficacy of cationic photosensitisers against Gram-positive organisms is thought to be due to significant interaction between the cation and anionic moieties in the Gram-positive cell wall, for example the well-documented interaction between dimethyl Methylene Blue and staphylococcal teichoic acid [12]. The disruption of membranes by organic cations is well established [13] and presumably contributes to the dark toxicities of the pheno-

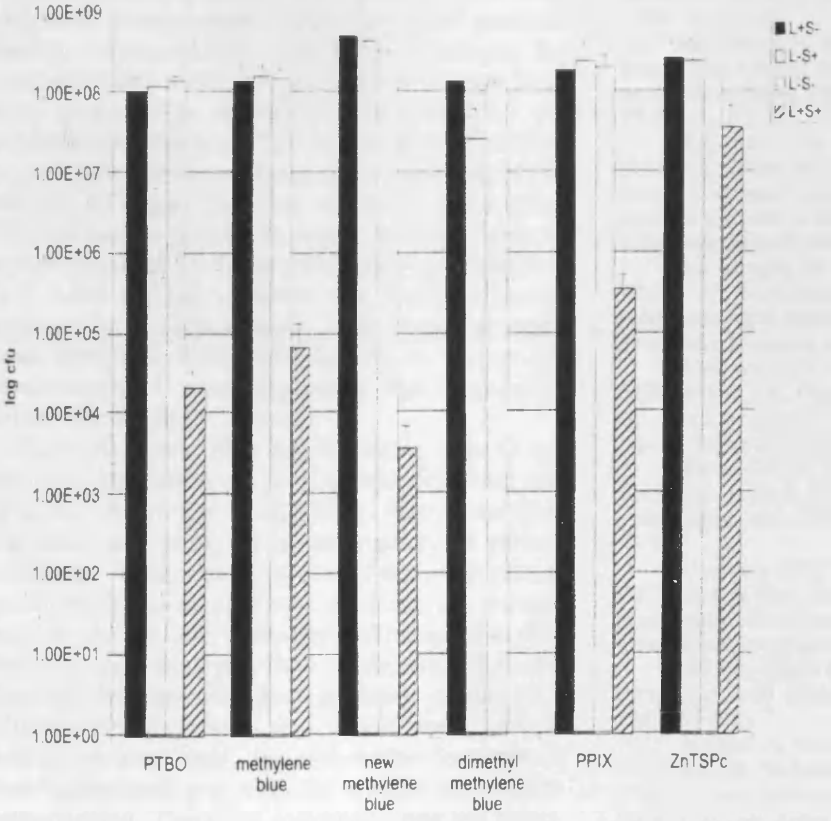


FIGURE 2 - Photobactericidal effects of the photosensitisers. Bacteria were either exposed to 21.8 J cm^{-2} of laser light in the presence of the sensitiser at a concentration of 40.85 µM (L+S+) or were kept in the dark in the absence of the sensitiser (L-S-). Additional controls consisted of bacteria irradiated with laser light in the absence of the sensitiser (L+S-) and bacteria kept in the dark in the presence of the sensitiser (L-S+). Bars represent mean values of the viable counts and error bars represent standard deviations.

thiazinium compounds in the present study. Such work has been extended by others to include photosensitisers with polycationic side chains which are thought to disrupt the plasma membrane of Gram-negative bacteria [14]. Obviously, such interactions cannot occur with anionic photosensitisers, possibly the activity of these compounds relying on lipophilic character to allow some partitioning of the photosensitiser into the plasma membrane. Noticeably, the anionic photosensitisers PPIX and ZnTSPc were the poorest photobactericidal agents of the series employed in the current study, with the highly hydrophilic ZnTSPc being the much less active of the two (*Figure 2*). In stating the significance of the anionic nature of the poorer photosensitisers here, it should be noted that a hydrophilic cationic zinc phthalocyanine derivative, bearing pyridinium moieties, has been reported to be an effective photobactericidal agent against *Escherichia coli* and *Staphylococcus aureus* [15]. The photobactericidal efficacy of the porphyrin derivative, chlorin e6, when conjugated to a cationic poly(lysine) side chain, is also well established [16].

As mentioned above, the likely multifactorial mode of photodynamic attack, including the activity of singlet oxygen against antioxidant enzymes, means that there is little chance of bacterial cell defence. Photodynamic antimicrobial agents thus offer a novel and highly effective treatment modality. Currently the main barrier to the clinical use of photodynamic antimicrobials is their perceived potential toxicity /mutagenicity in humans. However, the photosensitisers employed in the current study have been shown to be reasonable candidates for the photodynamic therapy (PDT) of cancer and exhibited sufficient differential (light:dark) mammalian cell toxicities to suggest their use in clinical disinfection. This has been supported in recent work by Zeina *et al.* for the use of Methylene Blue and Toluidine Blue O [17]. It has also been reported that the lethal photosensitisation of bacteria using TBO occurs at much lower light doses than those leading to human cell phototoxicity [18], again suggesting that therapeutic differentials might be achieved.

Both Methylene Blue and Toluidine Blue O are approved for clinical use (e.g. by the US Food and Drug Administration). In addition, Methylene Blue has been used safely for a wide variety of clinical conditions [19] and, indeed, is one of the most investigated medicinal compounds in existence, having been in use for over a century. Although the phenothiazinium congeners New Methylene Blue and dimethyl Methylene Blue both exhibited greater photobactericidal efficacies in the current study, particularly in the latter case, the mammalian toxicities of these compounds are presently unclear and require further testing. There are indications that the toxicities are significantly higher than those of Methylene Blue and Toluidine Blue O [20,21].

Given that photobactericidal agents are only proposed for use in topical therapy / disinfection, the efficacy and low toxicities of Methylene Blue and Toluidine Blue O again recommend their use in this field over that of anionic photosensitisers. From a healthcare economics viewpoint, these two agents are also suggested on the grounds of low cost and ready availability, which might promote their use - at least in the short term – over bioconjugates and antibody-linked photosensitisers.

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REFERENCES

[1] Gonzales RD, Schreckenberger PC, Graham MB, Kelkar K, DenBesten K, Quinn JP. Infections due to vancomycin-resistant *Enterococcus faecium* resistant to linezolid. Lancet 2001; 357: 1179.

[2] UK Department of Health. The Path of Least Resistance, HMG 1998

[3] Wainwright M. Photodynamic antimicrobial chemotherapy (PACT). J Antimicrob Chemother 1998; 42: 13-28.

[4] Kim SY, Kwon OJ, Park JW. Inactivation of catalase and superoxide dismutase by singlet oxygen derived from photoactivated dye. Biochimie 2001; 83: 437-444.

[5] Wilson M, Dobson J, Harvey W. Sensitization of oral bacteria to killing by low-power laser radiation. Curr Microbiol 1992; 25: 77-81.

[6] Wainwright M, Phoenix DA, Marland J, Wareing DRA, Bolton FJ. A study of photobactericidal activity in the phenothiazinium series. FEMS Immunol Med Microbiol 1997; 19: 75-80.

[7] Banks J, Poole S, Nair SP, Lewthwaite J, Tabona P, McNab R, Wilson M, Paul A, Henderson B. *Streptococcus sanguis* secretes CD14-binding proteins that stimulate cytokine synthesis: a clue to the pathogenesis of infective (bacterial) endocarditis? Microb Pathogen 2002; 32: 105-116.

[8] Wainwright M, Phoenix DA, Rice L, Burrow SM, Waring JJ. Increased cytotoxicity and phototoxicity in the methylene blue series via chromophore methylation. J Photochem Photobiol B Biol 1997; 40: 233-239.

[9] Wainwright M, Phoenix DA, Laycock SL, Wareing DRA, Wright PA. Photobactericidal activity of phenothiazinium dyes against methicillin-resistant strains of *Staphylococcus aureus*. FEMS Microbiol Lett 1998; 160: 177-181.

[10] Wainwright M, Phoenix DA, Marland J, Wareing DRA, Bolton FJ. A study of photobactericidal activity in the phenothiazinium series. FEMS Immunol Med Microbiol 1997; 19: 75-80.

[11] Usacheva MN, Teichert MC, Biel MA. Comparison of the Methylene Blue and Toluidine Blue photobactericidal efficacy against Gram-positive and Gram-negative microorganisms. Lasers Surg Med 2001; 29: 165-173.

[12] Pal MK, Ghosh TC. Binding of dyes by teichoic acid in competition with other polyanions. Makromol Chem 1991; 192: 467-471.

[13] Minnock A, Vernon DI, Schofield J, Griffiths J, Parish JH, Brown SB. Mechanism of uptake of a cationic water-soluble pyridinium zinc phthalocyanine across the outer membrane of *Escherichia coli*. Antimicrob Chemother 2000; 44: 522-527.

[14] Soukos NS, Ximenez-Fyvie LA, Hamblin MR, Socransky SS, Hasan T. Targeted antimicrobial photochemotherapy. Antimicrob Chemother 1998; 42: 2595-2601.

[15] Minnock A, Vernon DI, Schofield J, Griffiths J, Parish JH, Brown SB. Photoinactivation of bacteria. Use of a cationic water-soluble zinc phthalocyanine to photoinactivate both Gram-negative and Gram-positive bacteria. J Photochem Photobiol B 1996; 32: 159-164.

[16] Hamblin MR, O'Donnell DA, Murthy N, Rajagopalan K, Michaud N, Sherwood ME, Hasan T. Polycationic photosensitizer conjugates: effects of chain length and Gram classification on the photodynamic inactivation of bacteria. J Antimicrob Chemother 2002; 49: 941-951.

[17] Zeina B, Greenman J, Purcell WM, Das B. Killing of cutaneous microbial species by photodynamic therapy. Br J Dermatol 2001; 144: 274-278.

[18] Soukos NS, Wilson M, Burns T, Speight PM. Photodynamic effects of toluidine blue on human oral keratinocytes and fibroblasts and *Streptococcus sanguis* evaluated *in vitro*. Lasers Surg Med 1996; 18: 253-259.

[19] Wainwright M, Crossley KB. Methylene Blue - a therapeutic dye for all seasons? J Chemother 2002; 14: 431-443.

[20] Wainwright M, Phoenix DA, Rice L, Burrow SM, Waring JJ. Increased cytotoxicity and phototoxicity in the methylene blue series *via* chromophore methylation. J Photochem Photobiol B Biol 1997; 40: 233-239.

[21] Rice L, Wainwright M, Phoenix DA. Phenothiazine photosensitizers III. Activity of methylene blue derivatives against pigmented melanoma cell lines. J Chemother 2000; 12: 94-104.